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(54) Title: SOLID LIPID PARTICLES, PARTICLES OF BIOACTIVE AGENTS AND METHODS FOR THE MANUFACTURE AND USE THEREOF (57) Abstract <p>The present invention is in the area of administration forms and delivery systems for drugs, vaccines and other biologically active agents. More specifically the invention is related to the preparation of suspensions of colloidal solid lipid particles (SLPs) of predominantly anisometrical shape with the lipid matrix being in a stable polymorphic modification and of suspensions of micron and submicron particles of bioactive agent (PBAs); as well as to the use of such suspensions or the lyophilizates thereof as delivery systems primarily for the parenteral administration of preferably poorly water-soluble bioactive substances, particularly drugs, and to their use in cosmetic, food and agricultural products. The emulsification process includes melting of the agent (a lipid and for bioactive agent) and mixing with an aqueous phase (heated to melt temperature). The emulsification is completed by a high-pressure homogenization.</p>		

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SOLID LIPID PARTICLES, PARTICLES OF BIOACTIVE AGENTS AND METHODS FOR THE MANUFACTURE AND USE THEREOF

This invention relates to suspensions of particles of biodegradable lipids solid at room temperature, preferably triglycerides, which can be used as carriers for poorly water soluble drugs or other bioactive agents, and to suspensions of particles constituted by biologically active agents such as drugs, insecticides, fungicides, pesticides, herbicides and fertilizers, as well as to the lyophilizates thereof. Both systems can be prepared by a melt emulsification process.

The properties of the solid lipid particles (SLPs) include biodegradability, avoidance of toxicologically active residues from the production process, enhanced physicochemical stability with regard to coalescence and drug leakage, modified surface characteristics, controlled release of incorporated substances and modified biodistribution. The particles can be prepared by an emulsification process of molten material creating liquid droplets which form crystalline anisometrical particles on cooling. The anisometrical particles are of micron and submicron size, predominantly in the size range from 50 to 500 nm. The described suspensions have several advantages over other drug carrier systems deriving from the solid biodegradable matrix being predominantly present in a β -polymorphic modification (e.g. β' , β_1 , β_2), and not in an amorphous or α -crystalline state.

The preparation of micron and submicron particles consisting of poorly water-soluble bioactive agents (PBAS) by emulsification of the molten substance presents a novel process to reduce the particle size and/or to modify the surface characteristics of powdered substances which can be accomplished by inexpensive techniques and by the use of physiologically acceptable additives only. The suspensions of the particles are an easy-to-handle product from the security point of view. The particles of bioactive agents provide for the modified biodistribution and bioavailability of the formulated drugs or other bioactive substances which implies a modification of the extent and rate of dissolution and absorption, the circulation time, the site of action and the way of disposition of the drug or

other bioactive substance. A reduction in particle size below the micrometer range provides for the direct intravenous administration of particles made from poorly water-soluble drugs without the need of a carrier vehicle.

5 FIELD OF THE INVENTION

The present invention is in the area of administration forms and delivery systems for drugs, vaccines and other biologically active agents such as insecticides, fungicides, pesticides, herbicides and fertilizers. More specifically, the invention is related to the preparation of suspensions of
10 colloidal solid lipid particles (SLPs) with the lipid matrix being in a stable polymorphic modification and of suspensions of micron and submicron particles of bioactive agents (PBAs), as well as to the use of such suspensions or the lyophilizates thereof as delivery systems, primarily for the parenteral but also for the peroral, nasal, pulmonary, rectal, dermal and
15 buccal administration of preferably poorly water-soluble bioactive substances, particularly drugs; and to their use in cosmetic, food and agricultural products. These suspension systems provide for the controlled release of incorporated or constituting substances as well as for the modified biodistribution and bioavailability of incorporated or constituting drugs,
20 which implies a modification of the extent and rate of dissolution and absorption, the circulation time, the site of action and the way of disposition of the drug.

BACKGROUND OF THE INVENTION

25 The parenteral, in particular the intravenous administration of water-insoluble or poorly water-soluble substances such as drugs or other biological materials often presents a problem to the formulator. Since the diameter of the smallest blood capillaries is only a few microns the intravenous application of larger particles would lead to capillary blockage. Solid
30 drug substances are, however, commonly disintegrated by milling and grinding, thereby generating particles from a few millimeters down to the micrometer size range which are too large to be injected directly as an aqueous suspension. As a consequence, intravenous administration

systems containing suspended particles of water-insoluble drugs are not commercially available due to the risk of embolism. A further decrease in particle size is expensive, ineffective or even impossible by conventional techniques. Additionally, the reduction of solids to submicron-sized powders
5 brings about heavy difficulties in handling these dry products such as an increased risk of dust explosions and cross-contamination problems in a factory environment. Moreover, such systems present a health risk for persons exposed to the possible inhalation and absorption of potent bio-active materials. Up to now the only possibilities to administer poorly
10 water-soluble substances by the intravenous route are the use of co-solvents or the development of carrier systems which incorporate such substances in vehicles with hydrophilic surfaces.

Basic requirements of an ideal drug carrier system imply biodegradability, non-toxicity and non-immunogenicity. Moreover, the carrier should be
15 suitable for the intended route of administration, e g with regard to particle size. Often a controlled release of the incorporated bioactive material is desired, for example when constant serum levels should be maintained over a long period of time or when the drug exhibits only a low therapeutic index.

Furthermore, carrier systems can be employed to prolong the half-life
20 of certain substances which are unstable due to rapid enzymatic or hydrolytic degradation in biological milieu. On the other hand the incorporation of drug in the carrier material also presents an opportunity to protect the host from the drug in case of non-selective toxic substances such as antitumour agents.

25 In many cases drug carrier systems are developed with the object to deliver drugs to site-specific targets under circumvention of uptake by the reticuloendothelial system (RES). The rationale for such a drug targeting is an enhancement of the drug's therapeutic efficacy by an increase of the drug concentration at the target site with a simultaneous decrease at non-
30 target sites, thereby rendering possible a reduction of the administered dose. Thus, the toxicity of drugs, e g anticancer agents, can be diminished, leading to a decrease of side effects.

The prerequisite of a successful site-specific delivery implies a certain selectivity of the carrier system for the target tissue as well as the accessibility of the desired target site. Targeting by the intravenous route of application is generally connected to an avoidance or at least a reduction of carrier uptake by the RES except for the cases where a direct targeting to cells of the RES is desired. Clearance of colloidal particles by the RES has been described to depend on particle size as well as on particle surface characteristics such as surface charge and surface hydrophobicity. In general, small particles are cleared less rapidly from the blood stream than large particles whereas charged particles are taken up more rapidly than hydrophilic non-charged particles. Due to these facts approaches to drug targeting are the modification of surface characteristics and the reduction of particle size.

Moreover, a small particle size is also required for the targeting of drugs to extravascular sites since extravasation is only feasible through a receptor-mediated uptake by phagocytosis/pinocytosis or where the endothelial wall is fenestrated. These fenestrations can be found for example in the sinusoids of liver, spleen and bone-marrow and show diameters of up to approximately 150 nm.

From the manufacturing point of view the ideal drug carrier system should be preparable without complications by easy-to-handle techniques in a reproducible manner and possibly at low production costs. The formulation should exhibit sufficient stability during preparation as well as on storage.

In recent years several colloidal systems have received special interest for their potential application as drug carriers, among them being liposomes, lipid emulsions, microspheres and nanoparticles. However, all of the systems mentioned possess a certain number of draw-backs which so far have prevented the break-through of any such system as a wide-spread, commercially exploited drug carrier.

Drug carrier systems in the micrometer size range are represented by microspheres consisting of a solid polymer matrix, and microcapsules in which a liquid or a solid phase is surrounded and encapsulated by a

polymer film. Nanoparticles consist, like microspheres, of a solid polymer matrix. Their mean particle size, however, lies in the nanometer range. Both micro- and nanoparticles are generally prepared either by emulsion polymerization or by solvent evaporation techniques. Due to these production methods micro- and nanoparticles bear the risk of residual contaminations from the production process like organic solvents such as chlorinated hydrocarbons, as well as toxic monomers, surfactants and cross-linking agents, which may lead to toxicological problems. Moreover, some polymeric materials such as polylactic acid and polylactic-glycolic acid degrade very slowly in vivo so that multiple administration could lead to polymer accumulation associated with adverse side effects. Other polymers such as polyalkylcyanoacrylates release toxic formaldehyde on degradation in the body.

Drug carrier systems for parenteral administration based on lipids are liposomes and submicron lipid emulsions. Although such systems consist of physiological components only, thus reducing toxicological problems there is a number of disadvantages associated with these lipid carriers.

Liposomes are spherical colloidal structures in which an internal aqueous phase is surrounded by one or more phospholipid bilayers. The potential use of liposomes as drug delivery systems has been disclosed inter alia in the U.S. Pat Nos 3,993,754 (issued Nov 23, 1976 to Rahmann and Cerny), 4,235,871 (issued Nov 25, 1980 to Papahadjopoulos and Szoka) and 4,356,167 (issued Oct 26, 1982 to L. Kelly). The major drawbacks of conventional liposomes are their instability on storage, the low reproducibility of manufacture, the low entrapment efficiency and the leakage of drugs.

According to the IUPAC definition, in an emulsion liquids or liquid crystals are dispersed in a liquid. Lipid emulsions for parenteral administration consist inter alia of liquid oil droplets, predominantly in the sub-micron size range, dispersed in an aqueous phase and stabilized by an interfacial film of emulsifiers. Typical formulations are disclosed in the Jap. Pat. No 55,476/79 issued May 7, 1979 to Okamota, Tsuda and Yokoyama. The preparation of a drug containing lipid emulsion is described in WO

91/02517 issued March 7, 1991 to Davis and Washington. The susceptibility of these lipid emulsions towards the incorporation of drugs is relatively high due to the mobility of drug molecules within the internal oil phase since diffusing molecules can easily protrude into the emulsifier film causing instabilities which lead to coalescence. Furthermore, release of incorporated drugs from lipid emulsions is relatively fast so that the possibilities for a sustained drug release are limited.

Fountain et al (U.S. Pat. No 4,610,868 issued Sept. 9, 1986) developed lipid matrix carriers which are described as globular structures of a hydrophobic compound and an amphiphatic compound with diameters from about 500 nm to about 100.000 nm. The hydrophobic compound can be liquid or solid. The preparation techniques, however, employ organic solvents and are thus associated with the problem of complete solvent removal.

So-called lipospheres disclosed by Domb et al (U.S. Pat. Appl. No 435,546 lodged Nov 13, 1989; Int. Appl. No PCT/US90/06519 filed Nov 8, 1990) are described as suspensions of solid, water-insoluble microspheres made of a solid hydrophobic core surrounded by a phospholipid layer. Lipospheres are claimed to provide for the sustained release of entrapped substances controlled by the phospholipid layer. They can be prepared by a melt or by a solvent technique, the latter creating toxicological problems if the solvent is not completely removed.

A slow release composition of fat or wax and a biologically active protein, peptide or polypeptide suitable for parenteral administration to animals is disclosed in U.S. Pat. Appl. No 895,608 lodged Aug 11, 1986 to Staber, Fishbein and Cady (EP-A-0 257 368). The systems are prepared by spray drying and consist of spherical particles in the micrometer size range up to 1.000 microns so that intravenous administration is not possible.

Problems with the formulation of water-insoluble or poorly water-soluble substances are not restricted to the parenteral route of administration. Thus, the peroral bioavailability of drugs is related to their solubility in the gastrointestinal tract (GIT), and it is generally found that poorly water-soluble drugs exhibit a low bioavailability. Moreover, the dissolution of

drugs in the GIT is influenced by their wettability. Substances with apolar surfaces are scarcely wetted in media so that their dissolution rate is very slow.

5 In an attempt to improve the intestinal absorption of lipophilic drugs, Eldem et al (Pharm. Res. 8, 1991, 47-54) prepared lipid micropellets by spray-drying and spray-congealing processes. The micropellets are described as spherical particles with smooth surfaces. The lipids are, however, present in unstable polymorphic forms, and polymorphic phase transitions occur during storage so that the product properties are constant-
10 ly changing (T. Eldem et al, Pharm. Res. 8, 1991, 178 -184). Thus, constant product qualities cannot be assured.

Lipid nanopellets for peroral administration of poorly bioavailable drugs are disclosed in EP 0 167 825 of Aug 8, 1990 to P. Speiser. The nanopellets represent drug-loaded fat particles solid at room temperature
15 and small enough to be persorbed. Persorption is the transport of intact particles through the intestinal mucosa into the lymph and blood compartment. The lipid nanopellets are prepared by emulsifying molten lipids in an aqueous phase by high-speed stirring. After cooling to room temperature the pellets are dispersed by sonication.

20

OBJECT OF THE INVENTION

Considering the limitations of conventional drug carriers such as liposomes, lipid emulsions, nanoparticles and microspheres as outlined above there is an obvious demand for a carrier system for the controlled
25 delivery of poorly water-soluble bioactive substances to circumvent the drawbacks of traditional systems particularly with regard to preparation, stability, toxicity and modification of biodistribution.

The present invention introduces a new type of carrier system characterized as non-spherically shaped particles composed of crystalline
30 lipids, preferably triglycerides, and physiologically acceptable additives as well as a process for the manufacturing thereof. These carriers provide for the controlled delivery of poorly water-soluble substances such as drugs or other biological materials primarily by the parenteral but also by the peroral,

nasal, pulmonary, rectal, dermal and buccal route of administration, and will hereinafter be referred to as solid lipid particles (SLPs).

SLPs are characterized as lipidic particles of a solid physical state in the micro- and predominantly in the nanometer size range. The shape of the particles is mainly anisometric which is a consequence of the matrix forming lipids present in a β -polymorphic modification (e.g. β' , β_1 , β_2), and not in an amorphous or α -crystalline state. The properties of the SLPs include: (1) biodegradability and non-toxicity; (2) the ability to incorporate poorly water-soluble substances; (3) improved chemical and physical stability; (4) the possibility to prepare a dry storage formulation; (5) control of release characteristics of incorporated substances; and (6) modified surface characteristics. As a result of these properties SLPs overcome many of the problems encountered with conventional drug carrier systems.

The present invention is supposed to bring about the following advantages as derived from the characteristics of the SLPs described above:

- (1) SLPs can be prepared of biodegradable, pharmacologically acceptable compounds only and are, therefore, non-toxic. Additionally, the preparation of SLPs avoids the employment of organic solvents or any other potentially toxic additives, thus evading the contamination of the product with residual impurities.
- (2) SLPs possess an enhanced chemical stability as compared with conventional lipid emulsions based on liquid triglyceride oils owing to the lower degree of unsaturated fatty acids of solid triglycerides. Moreover, SLPs exhibit a better physical stability due to the solid nature of the lipid matrix which is expectedly more resistant to coalescence than fluid emulsion droplets. Furthermore, the lipid matrix is present in a stable β -polymorphic modification (e.g. β' , β_1 , β_2). Thus, the product properties will not change significantly during long-term storage due to polymorphic transformations.

- 5 (3) Suspensions of SLPs can be lyophilized by freeze-drying to provide a water-free storage system that exhibits a good long-term stability. The lyophilized powder can be redispersed in water, buffer or solutions of amino acids, carbohydrates and other infusion solutions directly before use or can be processed into other pharmaceutical formulations.
- 10 (4) Due to their lipophilic nature SLPs are suited for the solubilization of lipophilic and poorly water-soluble substances by entrapment into the lipid matrix. Compared to lipid emulsions SLPs are supposed to be less sensitive to the incorporation of drugs or other bioactive materials due to their solid nature. Drugs or other bioactive materials diffusing into the emulsifier film or recrystallizing close to the surface perturb the stabilizing film of emulsion droplets, increasing the risk of film rupture followed by coalescence. In contrast, film elasticity and film viscosity are of minor importance in the case of solid suspension particles since they cannot coalesce because of the rigid nature of the lipid.
- 15 (5) Drug release from the lipid carrier can be controlled for example by the composition of the lipid matrix by the choice of stabilizing agents as well as by the size of SLPs. Drug leakage is hindered by the solid state of the carrier due to the restricted drug diffusion.
- 20 (6) Drugs or bioactive substances exhibiting a short half-life due to enzymatic or hydrolytic degradation can be protected from rapid decomposition by incorporation within the lipid carrier since the hydrophobic matrix prevents the access of water to the incorporated drug on storage as well as in body fluids.
- 25 (7) The incorporation into SLPs of drugs or other bioactive substances with a low bioavailability due to poor solubility in the gastrointestinal tract (GIT) can enhance the bioavailability of such substances because these are solubilized in the
- 30

biodegradable lipid matrix and are thus present in the dissolved state.

(8) Due to the anisometrical shape of SLPs the specific surface area is larger than that of spherical particles of the same volume. Substances with a low peroral bioavailability can be absorbed faster and to a higher degree in the GIT when they are incorporated in anisometrical SLPs than in spherical lipid particles of the same volume, due to the larger surface area of SLPs since the potential site of action for lipolytic enzymes is larger.

(9) The surface characteristics of SLPs can be modified by variation of the lipid composition, use of different stabilizers, exchange of surfactants and/or adsorption of polymeric compounds. The modification of surface characteristics brings about the possibility to modify the in vivo distribution of the carrier and the incorporated substance. In case of intravenous administration this implies a modified uptake by the RES with the potential for drug targeting.

(10) Due to the submicron particle size of SLPs there is no risk of embolism by parenteral administration. Since SLPs can be prepared down to a particle size of about 50 nm, they possess the opportunity of extravasation through fenestrations of the endothelial wall. Thereby, drugs can be targeted to extravascular sites such as the bone marrow, for example.

Furthermore, the present invention introduces a new type of delivery system for the parenteral, peroral, nasal, pulmonary, rectal, dermal and buccal administration of drugs or other bioactive substances as well as the process for the manufacturing thereof. These formulations are suspensions of particles formed by bioactive substances with modified surface characteristics and/or a reduced particle size as compared to the powdered substance, and will hereinafter be referred to as particles of bioactive agents (PBAs). The preparation of PBAs can avoid the employment of any

toxicologically active additives such as organic solvents or toxic monomers, and can be accomplished by easy-to-handle techniques.

PBAs can be used in the following fields of application:

- 5 a) as a parenteral delivery system with modified biodistribution for sparingly water-soluble bioactive substances without the need of a carrier vehicle;
- b) as a delivery system according to a) for peroral, nasal, pulmonary, rectal, dermal and buccal administration;
- 10 c) as a formulation for the peroral administration of drugs with a poor bioavailability due to a low dissolution rate in the gastrointestinal tract;
- d) as a delivery system for use in agricultural applications;
- e) the lyophilizate of formulations a) to d) as a reconstitutable powder with an enhanced stability on storage.

15 Owing to the special characteristics of the present invention PBAs are supposed to bring about the following advantages over conventional pharmaceutical delivery systems:

- 20 1) The formulation of poorly water-soluble drugs or other bioactive substances as micron and submicron particles avoids the need of a carrier system for their parenteral application, thereby circumventing the disadvantages of conventional drug carriers like liposomes, lipid emulsions, nanoparticles and microspheres.
- 25 2) PBAs can be prepared by easy-to-handle techniques in a reproducible way. There are no problems to foresee for the scaling up of the manufacturing process.
- 30 3) Since the particles consist of the pure bioactive compound with only small amounts of stabilizers the drug-load capacity of the drug particles is high.
- 4) The release of drugs or other bioactive compounds from the formulation can be controlled by the choice of amphiphatic compounds employed to stabilize the particles.
- 5) The preparation of PBAs can avoid the use of toxicologically active additives.

- 6) A water-free storage system with enhanced stability can be produced, for example by freeze-drying of the PBA dispersions.
- 7) The surface characteristics of PBAs can be modified by the choice of amphiphatic compounds used as stabilizers as well as by the attachment of so-called homing devices for the targeting of drugs, for example monoclonal antibodies or carbohydrate moieties. The surface modifications give rise to a modified bioavailability and biodistribution with regard to the extent and rate of absorption, the circulation time, the site of action and the way of disposition of the bioactive substance. The modification of surface characteristics also provides the opportunity to avoid or at least to reduce the uptake of intravenously administered particles by cells of the RES.
- 8) Since the particles can be prepared with a size below 100 nm to 200 nm they possess the opportunity for extravasation by fenestrations of the endothelial wall. Thereby, drugs can be targeted to extravascular sites such as the bone-marrow, for example.
- 9) A reduction in particle size to the nanometer size range generally not achievable by milling or grinding leads to an enormous increase of the specific surface area of the particles. Since the peroral bioavailability of drugs or other bioactive substances is related to the specific surface area via the dissolution rate of the substance in the gastrointestinal tract the submicron sized particles give rise to an enhanced bioavailability of drugs poorly soluble in the GIT.
- 10) Hydrophobic substances can be formulated as PBAs with hydrophilic surfaces. Hydrophilic surfaces provide for a good wettability of the particles, for example in the GIT, facilitating dissolution of the compound. Thus, the bioavailability can be increased.
- 11) The process of manufacturing of PBAs involves inexpensive easy-to-handle techniques only and provides a product which is safe to handle. Since the particles are present in a liquid dispersion there is no risk of dust explosions, cross-contamination or inhalation of bioactive substances as often encountered with the production of extremely fine powders.

DESCRIPTION OF THE INVENTION

The present invention relates to suspensions of micron and sub-micron particles of biodegradable lipids solid at room temperature (solid lipid particles, SLPs), to suspensions of particles of meltable bioactive substances (PBAs), to lyophilizates thereof and to methods for the manufacturing thereof.

Solid lipid particles (SLPs) are of predominantly anisometrical shape which is a consequence of the lipid matrix being present in a β -polymorphic modification (e.g. β' , β_1 , β_2) or in a polymorphic state analogous to that of β -crystals of triglycerides and not in an amorphous or α -crystalline-like state. SLPs can be used as carrier systems primarily for the parenteral but also for the peroral, nasal, pulmonary, rectal, dermal and buccal administration of poorly water-soluble substances such as drugs or other biologically active materials. The application of SLPs is, however, not restricted to the administration of pharmaceuticals to humans or animals. SLPs can also be used in cosmetic, food and agricultural products. SLPs are novel lipid structures with properties that overcome many of the problems associated with previously described carrier systems.

The matrix of SLPs is constituted by biocompatible hydrophobic materials which are solid at room temperature and have melting points ranging from approximately 30 to 120°C. The preferred matrix constituents are solid lipids (fats) such as mono-, di- and triglycerides of long-chain fatty acids; hydrogenated vegetable oils; fatty acids and their esters; fatty alcohols and their esters and ethers; natural or synthetic waxes such as beeswax and carnauba wax; wax alcohols and their esters, sterols such as cholesterol and its esters, hard paraffins, as well as mixtures thereof. The carrier material must be compatible with the agent to be incorporated.

Lipids are known to exhibit a pronounced polymorphism. This can be defined as the ability to reveal different unit cell structures in crystal, originating from a variety of molecular conformations and molecular packings. Depending on the conditions, glycerides, for example, may crystallize in three different polymorphic forms termed alpha (α), beta prime (β') and beta (β) according to the classification of Larsson (K. Larsson, 1966, Acta Chem.

Scand. 20, 2255-2260). These polymorphic modifications characterized by a particular carbon chain packing may differ significantly in their properties such as solubility, melting point and thermal stability. Transformations take place from α to β' to β , the transition being monotropic. The β -form is the thermodynamically most stable polymorph, whereas α is the least stable and will transform more or less rapidly into the more stable polymorphs β' and β , depending on the thermal conditions. This transformation is accompanied by a change of physicochemical properties.

In the described suspensions of SLPs the lipid matrix is predominantly present in a stable polymorphic modification. Although on cooling, the dispersed melt metastable polymorphs such as the α -form may occur intermediately a stable polymorph is formed within several hours or days after preparation of the dispersions.

The suspensions of SLPs can be stabilized by amphiphatic compounds such as ionic and non-ionic surfactants. Suitable stabilizers include but are not limited to the following examples: naturally occurring as well as synthetic phospholipids, their hydrogenated derivatives and mixtures thereof, sphingolipids and glycosphingolipids; physiological bile salts such as sodium cholate, sodium dehydrocholate, sodium deoxycholate, sodium glycocholate and sodium taurocholate; saturated and unsaturated fatty acids or fatty alcohols; ethoxylated fatty acids or fatty alcohols and their esters and ethers; alkylaryl-polyether alcohols such as tyloxapol; esters and ethers of sugars or sugar alcohols with fatty acids or fatty alcohols; acetylated or ethoxylated mono- and diglycerides; synthetic biodegradable polymers like block co-polymers of polyoxyethylene and polyoxypropyleneoxide; ethoxylated sorbitanesters or sorbitanethers; amino acids, polypeptides and proteins such as gelatine and albumin; or a combination of two or more of the above mentioned.

The aqueous phase in which the SLPs are dispersed can contain water-soluble or dispersable stabilizers; isotonicity agents such as glycerol or xylitol; cryoprotectants such as sucrose, glucose, trehalose etc; electrolytes; buffers; antiflocculants such as sodium citrate, sodium pyrophosphate or sodium dodecylsulfate; preservatives.

Depending on the characteristics of the employed stabilizers the coexistence of other colloidal structures such as micelles and vesicles in suspensions of SLPs cannot be ruled out.

Substances particularly suitable for the entrapment into SLPs are
5 drugs or other bioactive compounds which are poorly water-soluble, show a low bioavailability, are badly absorbed from the intestine, and/or will be rapidly degraded in biological environment by chemical or enzymatical processes, as well as low-specific active substances which are highly toxic at non-target sites. In case it is desired to incorporate a relatively water-
10 soluble compound into SLPs it is necessary to decrease the water-solubility of this compound, which can be achieved for example by using a water-insoluble derivative of the compound such as an acid or base, a complex, or a lipophilic precursor.

Drugs or bioactive agents particularly suited for incorporation into
15 SLPs are antibiotics such as fosfomycin, fosmidomycin and rifapentin; antihypertensives such as minoxidil, dihydroergotoxine and endralazine; antihypotensives such as dihydroergotamine; systemic antimycotics such as ketoconazole and griseofulvin; antiphlogistics such as indomethacin, diclofenac, ibuprofen, ketoprofen and piroprofen; antiviral agents such as
20 aciclovir, vidarabin and immunoglobulines; ACE inhibitors such as captopril and enalapril; betablockers such as propranolol, atenolol, metoprolol, pindolol, oxprenolol and labetalol; bronchodilators such as ipratropium-bromide and salbutamol; calcium antagonists such as diltiazem, flunarizin, verapamil, nifedipin, nimodipin and nitrendipin; cardiac glycosides such as
25 digitoxin, digoxin, methyl digoxin and acetyldigoxin; cephalosporins such as ceftizoxim, cefalexin, cefalotin and cefotaxim; cytostatics such as chlor-methin, cyclophosphamid, chlorambucil, cytarabin, vincristin, mitomycin C, doxorubicin, bleomycin, cisplatin, taxol, penclomedine and estramustin; hypnotics such as flurazepam, nitrazepam and lorazepam; psychotropic
30 drugs such as oxazepam, diazepam and bromazepam; steroid hormones such as cortisone, hydrocortisone, prednisone, prednisolone, dexamethasone, progesterone, pregnanolone, testosterone and testosteroneundecanoate; vasodilators such as molsidomin, hydralazin and dihydralazin;

cerebral vasodilators such as dihydroergotoxin, ciclonicat and vincamin; lipophilic vitamins such as vitamins A, D, E, K and their derivates.

The bioactive substances can be located in the core of SLPs where they are dissolved, solubilized or dispersed in the matrix, and/or in the
5 stabilizer layer(s) surrounding the particle matrix, and/or can be adsorbed to the surface of SLPs. The bioactive substances can be dissolved or crystalline or amorphous or a mixture of these crystallographic states.

SLPs can be prepared by an emulsification process which exhibits certain similarities to the preparation of lipid(oil)-in-water emulsions but is
10 mainly characterized by its basic differences as will be outlined below. The process is described as follows:

- (1) The solid lipid or the mixture of lipids is melted.
- (2) The stabilizers are added either to the lipid and to the dispersion medium or to the dispersion medium only, depending on their
15 physicochemical characteristics. The choice of stabilizers and the admixture regime are not comparable with those applied for lipid (oil)-in-water emulsions, which is evident from the below examples. Stabilizers may also be added or exchanged after homogenization, for example by adsorption of polymers or by dialysis of water-soluble
20 surfactants.
- (3) Drugs or other bioactive substances to be incorporated into the SLPs may be melted together with the lipids if the physicochemical characteristics of the substance permit, or may be dissolved, solubilized or dispersed in the lipid melt before homogenization.
- (4) The dispersion medium is heated to the temperature of the melt
25 before mixing and may contain for example stabilizers, isotonicity agents, buffering substances, cryoprotectants and/or preservatives.
- (5) The melted lipid compounds are emulsified in the dispersion medium, preferably by high pressure homogenization, but emulsification is
30 also possible by sonication, high speed stirring, vortexing and vigorous hand shaking. The way of homogenization determines the particle size of the SLPs.

The basic differences to the preparation of lipid-in-water emulsions beside the choice and admixture regime of the stabilizers are related to the following steps:

- 5 (6) After homogenization the dispersion can be sterilized by standard techniques such as autoclaving or filtration through a 0.2 μm sterile filter provided the particles are small enough not to be retained by the filter. These steps have to be performed before the system is cooled below the recrystallization temperature. Moreover, contaminations which could lead to heterogenous nucleation should be avoided.
- 10 It is therefore advisable to remove particulate contaminations from the dispersions by filtration prior to cooling below the recrystallization temperature. The pore size of the filter should be chosen sufficiently large so as not to retain the lipid particles.
- 15 (7) The dispersions are allowed to stand to cool off at room temperature forming SLPs by recrystallization of the dispersed lipids. During cooling the dispersion may be agitated by a magnetic stirrer for example.
- 20 (8) In a subsequent step the dispersion medium is reduced in volume for example by evaporation or it can be removed by standard techniques such as filtration, ultrafiltration or freeze-drying, thus yielding a water-free storage system which can be reconstituted prior to use. The lyophilized powder can also be processed into other pharmaceutical, cosmetic, food or agricultural formulations such as powders, tablets, capsules etc.
- 25 SLPs are typically solid particles of anisometrical shape as demonstrated by Fig. 1 which shows a transmission electron micrograph of a freeze-fractured specimen of the SLPs of Example 1. The anisometrical particle shape results from crystallization of the lipid matrix in the β -polymorphic form. Solidification of the amorphous fat or crystallization of the
- 30 unstable α -polymorph generally reveals spherical particles. The presence of the stable β -form could be detected by differential scanning calorimetry (Fig. 2) and synchrotron radiation wide-angle X-ray diffraction (Fig. 3).

The particle size of SLPs depends on the type and amount of emulsifier and on the emulsification technique and conditions (see below). The resolidification of the molten lipids prior to homogenization should be avoided because size reduction by homogenization is substantially impeded if the particles are solid during this step. To ensure that the molten lipids do not solidify prior to homogenization, i.e. before smaller particles can be formed, the dispersion medium is heated to approximately the same temperature as the melt before the two phases are mixed so that the melt will not be cooled down by the addition of the dispersion medium.

SLPs in the nanometer size range are obtained by high-pressure homogenization. The particles show a relatively narrow particle size distribution with mean particle sizes by number of approximately 50 - 300 nm as determined by photon correlation spectroscopy (PCS). The dispersions of SLPs are stable on storage for more than 18 months. Thus, the long-term stability is similar to that of submicron o/w emulsions used for parenteral nutrition. Long-term stability data of other solid lipid-based carrier dispersions described in the patent literature such as lipospheres (A. Domb et al, Int. Appl. No PCT/US90/06519 filed Nov 8, 1990) and lipid nanopellets (P. Speiser, Eur. Pat. No 0167825 issued Aug 8, 1990) could not be found. Domb describes phospholipid stabilized tristearate lipospheres with a seven day stability as "exceptionally stable".

It turned out that the stabilization of SLP suspensions requires the presence of a highly mobile stabilizing agent in the dispersion medium in such a way that the amount of highly mobile stabilizers in the dispersion medium is, after emulsification, sufficient to stabilize newly created surfaces during recrystallization (see below). Bile salts, especially in combination with nonelectrolytic compounds such as glycerol in concentrations used to achieve blood isotony, have proved to be very efficient in this respect. SLPs stabilized by phospholipids alone, or in combination with nonelectrolytic compounds such as glycerol in concentrations used to achieve blood isotony, tend to form semi-solid ointment-like gels as shown on the transmission electron micrograph of Fig. 4, whereas the addition of sodium

glycocholate to the aqueous phase prevents this gel formation (B. Siekmann and K. Westesen, 1992, Pharm. Pharmacol. Lett. 1, 123-126).

5 A molar phospholipid to bile salt ratio between 2:1 and 4:1 turned out to be most effective regarding the initial stabilization during homogenization as well as the long-term storage stability of SLP dispersions. These phospholipid/bile salt ratios are above the ratio of formation of mixed micelles and coincide with a swollen lamellar phase of mixed lecithin/bile salt layers in the ternary phase diagram of the system lecithin/bile salt/water. Data therefore suggest that stabilization is most effective if the bile salt is not
10 bound to mixed micelles and that during stabilization of SLPs the bile salt molecules are inserted in the phospholipid layer on the surface of the particles.

SLPs can also be sterically stabilized by nonionic surfactants. Steric stabilization of SLPs requires, however, a relatively high amount of surfactants with lipid/surfactant ratios up to 1:1. It can be observed in general that
15 the stability of SLPs decreases with increasing lipid/surfactant ratio.

The amount of emulsifier required for surface stabilization of the dispersed particles is higher than in conventional lipid emulsions, for example such as used in parenteral nutrition. This effect can be attributed
20 to the crystallization of the molten lipids after homogenization. Since the lipids typically do not recrystallize or exist (on storage) in the form of ideal spheres but as anisometric particles there is a large increase in surface area as compared to the droplets of the emulsified molten lipids or of conventional lipid emulsions, respectively. The additional surfaces newly
25 generated during recrystallization or polymorphic transitions of the dispersed lipids need to be stabilized immediately on formation to avoid particle aggregation. Therefore, the preparation of stable SLP dispersions requires the presence of a reservoir of stabilizing agents after emulsification.

The choice of stabilizers cannot be deduced from compositions and
30 stabilization mechanisms for oil-in-water emulsions but is dependent on the existence of highly mobile stabilizers due to the formation mechanism of the anisometrical particles. In colloid and surface science "highly mobile" generally refers to free diffusion in the dispersion medium at a high diffusion

velocity. With regard to the stabilization of colloidal solid lipid particles the diffusion velocity should be sufficiently high to reach freshly created particle surfaces (especially during re-crystallization of the lipid) before particle aggregation can take place, in order to exert a stabilizing action at the lipid/water interface to prevent particle aggregation. Sufficiently high diffusional velocities are typically observed with substances which do not form a separate phase in the dispersion medium according to the phase rule set up by Gibbs. Highly mobile stabilizers can be of ionic or nonionic nature. Typically, these stabilizers dissolve molecularly in the dispersion medium and/or form micelles. Micelles are known to be highly dynamic structures characterized by a fast exchange of molecules between micellar aggregates and the dispersion medium. The monomers in the dispersion medium are immediately available for surface stabilization. In contrast, stabilizing agents that tend to form a separate phase in the dispersion medium are not sufficiently mobile to stabilize freshly created surfaces before particle aggregation can take place. These stabilizers are therefore not suitable as sole stabilizers of SLP dispersions. Phospholipids are an example of stabilizing agents that form a separate phase in the dispersion medium. It is well known that phospholipids form closed lamellar structures, so called vesicles, in aqueous media, and that the exchange rate of phospholipid molecules between vesicles and the aqueous phase is extremely small compared to that of micelles. Phospholipid molecules are therefore bound in vesicular structures and are not immediately available to stabilize newly created surfaces during recrystallization of the lipid particles. Consequently, phospholipids alone, although suitable as effective stabilizers of lipid emulsions, cannot efficiently stabilize SLP suspensions as is evident from Examples 5 and 13. In fact, preparing SLPs with a standard composition of lipid emulsions, for example 10% fat and 1.2% phospholipids, results in unstable SLP dispersions. Even higher concentrations of phospholipids such as 20% or 60% lecithin related to the fat phase are not sufficient to stabilize SLP dispersions as demonstrated in Example 5.

Although stated in the patent literature (e.g. Domb et al in U.S. Pat No 435,546 issued Nov 13, 1989 and Int. Appl. No PCT/US90/06519 filed

Nov 8, 1990), fine suspensions of solid lipids are not equivalent to sub-micron lipid emulsions in that respect that the inner phase is only replaced by solid fats instead of liquid ones. The physicochemical properties of lipid suspensions such as SLPs differ substantially from that of lipid emulsions.

5 As a consequence of these differences lipid suspensions cannot be prepared and treated analogously to lipid emulsions. One basic difference of SLPs is their much larger particle surface area as outlined above so that SLPs require a higher amount of surfactants which additionally need to be highly mobile to immediately stabilize the new surfaces created when the
10 molten lipid recrystallizes or transforms into the stable β -polymorph. The second basic difference is that once SLPs are formed by recrystallization of the molten lipid any renewed melting of the small particles may result in an instability of the dispersions if there is no excess of mobile stabilizer in the aqueous phase which is not adsorbed to particle surfaces where it is
15 immobilized. A further requirement for physicochemical stability of SLPs in contrast to oil-in-water emulsions is the absence of particulate impurities which could promote heterogenous nucleation. It is therefore advisable to remove particulate contaminations from the dispersions by filtration prior to cooling below the recrystallization temperature. Moreover, non-electrolytic
20 compounds used to achieve blood isotony such as glycerol turned out to promote the stability of SLP dispersions.

The present invention also relates to suspensions of particles of bioactive agents (PBAs). Sparingly water-soluble substances such as drugs, insecticides, fungicides, pesticides, herbicides, fertilizers, nutrients, cos-
25 metics etc which are meltable in the temperature range from approximately 30 to 120°C can be formulated as PBAs by a procedure similar to the preparation of SLPs as described above. The matrix of PBAs is constituted by the bioactive agent itself.

30 PBAs present a novel type of delivery system and can be characterized as predominantly submicron and/or micron particles of bioactive agents suspended in an aqueous media stabilized by amphiphatic compounds. PBAs possess modified surface characteristics which can be controlled by the choice of amphiphiles and/or a reduced particle size of the

matrix constituting compound as compared to the powdered substance. These characteristics give rise to a modified biodistribution and bioavailability of the formulated drugs or other bioactive substances which implies a modification of the extent and rate of dissolution and absorption, the circulation time, the site of action and the way of disposition of the drug or other bioactive substance. The physicochemical properties of PBAs depend strongly on the characteristics of the bioactive agent of which they are formulated, on the type and amount of stabilizing agents as well as on the way of emulsification. Suspensions and lyophilizates of PBAs can be used for the peroral, nasal, pulmonary, rectal, dermal, buccal and, depending on the particle size, also for the parenteral administration of poorly water-soluble drugs or other biologically active compounds. Moreover, PBAs can also be employed in cosmetic, food and agricultural products, in particular for the formulation of poorly water-soluble herbicides and pesticides.

The matrix of PBAs is constituted by practically insoluble or sparingly water-soluble agents with melting points preferably below 100°C or the melting points of which can be decreased to below 100°C by the addition of certain adjuvants. Substances particularly suitable for the formulation as PBAs are drugs or other bioactive materials which are poorly water-soluble, show a low bioavailability and/or are badly absorbed from the intestine. Examples of such substances comprise but are not limited to:

Anesthetics and narcotics such as butanilcaine, fencocaine, isobutambene, lidocaine, risocaine, prilocaine, pseudococaine, tetracaine, trimecaine, tropacocaine and etomidate; anticholinergics such as metixen and profenamine; antidepressives, psychostimulants and neuroleptics such as alimemazine, biperiden, perazine, chlorpromazine, fenpropionolol, fenpropionolol, fluanisone, mebenazine, methylphenidate, thioridazine, toloxatone and trimipramine; antiepileptics such as dimethadione and nicethamide; antimycotics such as butoconazole, chlorphenesin, etisazole, exalamid, pecilocine and miconazole; antiphlogistics such as butibufen and ibuprofen; bronchodilators such as bamifylline; cardiovascular drugs such as alprenolol, butobendine, cloridazole, hexobendine, nicofibrate, penbutolol, pirfenidone, prenylamine, procaine amide, propylthiouracil, suloctidil, toliprolol,

xibendol and viquidile; cytostatics such as asperline, chlorambucile, chlor-naphhazine, mitotane, estramustine, taxol, penclomedine and trofosfamide; hyperemic drugs such as capsaicine and methylnicotinate; lipid reducers such as nicoclonate, oxprenolol, pirifibrate, simfibrate and thiadenol; spas-
5 molytics such as aminopromazine, caronerine, difemerine, fencarbamide, tiopramide and moxaverine; testosterone derivates such as testosterone enantate and testosterone-(4-methylpentanoate); tranquilizers such as azaperone and buramate; virustatics such as arildon; vitamin A derivates such as retinol, retinol acetate and retinol palmitate; vitamin E derivates
10 such as tocopherol acetate, tocopherol succinate and tocopherol nicotinate; menadione; cholecalciferol; insecticides, pesticides and herbicides such as acephate, cyfluthrin, azinphosphomethyl, cypermethrine, substituted phenyl thiophosphates, fenclophos, permethrine, piperonal, tetramethrine and tri-fluraline.

15 As with SLPs, suspensions of PBAs can be stabilized by amphiphatic compounds. Principally the same ionic and nonionic surfactants which may be employed for the stabilization of SLPs are also suitable for the preparation of PBA suspensions. The choice of stabilizing agents depends on the physicochemical properties of both the bioactive substance and the disper-
20 sion medium as well as on the desired surface characteristics of the particles.

The aqueous phase in which the PBAs are dispersed should contain water-soluble (or dispersable) stabilizers; isotonicity agents such as glycerol or xylitol; cryoprotectants such as sucrose, glucose, trehalose etc; electro-
25 lytes; buffers; antiflocculants such as sodium citrate, sodium pyrophosphate or sodium dodecylsulfate; preservatives. Although water is the preferred dispersion medium the invention is, however, not restricted to aqueous dispersions alone but can be extended to any other pharmacologically acceptable liquid such as ethanol, propylene glycol and methyl-isobutyl-ketone, or a
30 mixture thereof.

Depending on the characteristics of the employed stabilizers the coexistence of other colloidal structures such as micelles and vesicles in suspensions of PBAs cannot be ruled out.

Suspensions of PBAs are typically prepared by an emulsification process similar to that of SLPs. The molten drug or bioactive substance or a mixture of such compounds is emulsified in a pharmacologically acceptable liquid immiscible with the melt, preferably by high-pressure homogenization. Emulsification is also possible by sonication, high-speed stirring, vortexing and vigorous hand shaking. The liquid is heated to the temperature of the melt before mixing and may contain for example isotonicity agents, buffering substances, cryoprotectants and/or preservatives.

The stabilizing amphiphatic compounds are added either to the melt and to the liquid or to the liquid only, depending on their physicochemical characteristics. Stabilizers may also be added or exchanged after homogenization, for example by the adsorption of polymers or by dialysis.

The PBAs manufactured according to the above described process can be categorized in two distinguishable groups.

The PBAs of the first group are characterized in that they are water-insoluble at the temperature of emulsion preparation and will not be solubilized by the excess of stabilizers or form micelles by themselves, the particle size of PBAs remaining unchanged before and after cooling to room temperature.

The PBAs of the second group are characterized in that they are partly water-soluble at the temperature of emulsion preparation and/or are able to form mixed micelles by the excess of stabilizers and/or form micelles by themselves, leading to an increase of particle size after cooling to room temperature due to, for example, crystal growth and/or precipitation of dissolved bioactive agent from the supersaturated solution and/or due to mass transport from smaller to larger particles, for example in micelles and/or by processes such as Ostwald ripening.

In a subsequent step the liquid phase can be removed by freeze-drying, for example, producing a reconstitutable powder which can also be processed into other pharmaceutical formulations.

PBAs are finely dispersed particles consisting of a matrix of bioactive material surrounded by one or more layers rich in surfactant. The particle size and the size distribution as well as the particle shape and the surfact-

ant coating depend on the properties and amounts of the matrix forming bioactive substances and the stabilizing agents, the ratio of bioactive material to amphiphatic compounds as well as on the way of emulsification.

5 EXAMPLES

Example 1: Method of preparation of tripalmitate SLPs.

In a thermostated vial 4.0 g tripalmitate (tripalmitin, 99% pure, Fluka) is heated to 75°C to melt the lipid. In the lipid melt 0.48 g soy bean lecithin (Lipoid S 100, Lipoid KG) is dispersed by probe sonication (MSE
10 Soniprep 150) until the dispersion appears optically clear. 0.16 g sodium glycocholate (glycocholic acid, sodium salt 99%, Sigma) and 4 mg thiomersal (Synopharm) is dissolved in 36 ml bidistilled water. The aqueous phase is heated to 75°C and added to the lipid melt. A crude dispersion is produced by probe sonication for approximately 2 minutes. The crude disper-
15 sion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 500 bar. Homogenization with this equipment is accomplished by extrusion through a small ring-shaped orifice. The homogenized dispersion is allowed to stand at room temperature to cool off. The dispersion
20 reveals trace amounts of visible fat particles which are separated from the dispersion by filtering it through a 0.45 µm sterile filter.

The importance of the admixture of highly mobile surfactants such as bile salts with regard to the particle size distributon and the stability of SLP dispersions is demonstrated below, e g in Examples 2, 5 to 7 and 13 to 15.

25 The mean particle size after preparation (by number) of the tripalmitate SLPs determined by photon correlation spectroscopy (PCS, Malvern Zetasizer 3) is 205 nm. After 15 months of storage the particles show no visible signs of aggregation, creaming, sedimentation or phase separation. A PCS multiangle measurement (Malvern Zetasizer 3, detection at five different angles: 50, 70, 90, 110 and 130 degrees) reveals a monomodal particle size distribution (by number) with a peak at 250 nm (Fig. 5).
30

At temperatures below the melting point of the lipid matrix tripalmitate SLPs are predominantly anisometrical particles as demonstrated in Fig. 1

which is a transmission electron micrograph of a freeze-fractured specimen of the tripalmitate SLPs of Example 1. Before preparation of the specimen the sample is stored at room temperature for 5 months. The sample is freeze-fractured at 173 K in a freeze-fracture unit BAF 400 (Balzers AG, CH-Liechtenstein). Fast freezing is accomplished by slush into melting propane. Shadowing of the sample is performed with platinum/carbon (layer thickness 2 nm) at 45 degrees and with pure carbon at 90 degrees for replica preparation. Replica are cleaned with a 1:1 (v/v) chloroform/ethanol mixture. Replica on uncoated grids are viewed with an electron microscope EM 300 (Philips, D-Kassel).

In the anisometrical tripalmitate particles the glyceride is present in the stable β -crystalline polymorph as indicated by thermoanalytical investigations. Fig. 2 presents a differential scanning calorimetric (DSC) thermogram of SLPs of Example 1 and of pure tripalmitate. The samples are weighed accurately into standard aluminium pans. Thermograms are recorded from 20°C to 90°C at a scan rate of 10°C/minute on a Perkin Elmer calorimeter DSC-7. The detected transition peaks correspond to the melting of tripalmitate β -crystals. The melting point of tripalmitate SLPs is shifted to a lower temperature compared to that of pure tripalmitate due to the presence of phospholipids and due to the small crystallite size.

Example 2: Method of preparation of isotonic tripalmitate SLPs.

7.0 g tripalmitate (tripalmitin, Fluka) is melted in a vial thermostated at 75°C. 840 mg soy bean lecithin (Lipoid S 100, Lipoid KG) is dispersed in the tripalmitate melt as described in Example 1. The aqueous phase containing 1.575 g glycerol, 280 mg sodium glycocholate and 4 mg thiomersal is heated to 75°C and added to the lipid melt to a weight of 70 g. A crude dispersion is produced by sonication for approximately 2 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 10 times through the homogenizer at a pressure of 800 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

The mean particle size by number of the isotonic tripalmitate SLPs determined by PCS is 125.9 nm after preparation and 116.2 nm after 50 days of storage, i.e. there was practically no particle growth. The slight deviations of the values fall into the range of accuracy of the sizing method.

5 The PCS particle size distribution is compared to that of the commercially available lipid emulsion for parenteral nutrition Intralipid™ in Fig. 6. Intralipid™ is composed of 10% soy bean oil, 1.2% fractionated phospholipids and 2.25% glycerol dispersed in water for injection. It can be observed that the particle size distribution of tripalmitate SLPs of Example 2 is significantly
10 smaller and more narrow than that of Intralipid™. In contrast to Example 1 the addition of glycerol results in a noticeable difference in the particle size distribution. Whereas the SLP dispersion of Example 1 contains trace amounts of visible suspension particles after being cooled to room temperature from the hot emulsion no macroscopically visible suspension particles
15 are observed in the dispersion of Example 2.

Investigations by synchrotron radiation wide-angle X-ray diffraction (Fig. 3) and differential scanning calorimetry reveal that the tripalmitate in SLPs is present in the stable β -polymorphic form at room temperature.

20 Example 3: Preparation of hard fat SLPs by microfluidization.

3.0 g hard fat (Witepsol™ W35, Hüls AG) is melted in a thermostated vial at 75°C. 1.8 g soy bean lecithin (Phospholipon 100, Natterman) is dispersed in the tripalmitate melt as described in Example 1. The aqueous phase containing 375 mg sodium glycocholate, 2.25 g glycerol and 10
25 mg thiomersal is heated to 75°C and added to the lipid melt to a weight of 100 g. A crude dispersion is produced by ultra-turrax vortexing for approximately 2 minutes. The crude dispersion is transferred to a microfluidizer (Microfluidics Microfluidizer M-110T), a high-pressure homogenizer of the jet-stream principle which is immersed in a thermostated water bath
30 (70°C). The dispersion is cycled through the microfluidizer for 10 minutes and allowed to stand at room temperature to cool off.

The mean particle size of hard fat SLPs after preparation is 45.9 nm as determined by PCS.

During homogenization a sample for particle sizing is drawn each minute in order to monitor the time course of homogenization. Fig. 7 displays the mean particle size versus homogenization time. The mean particle diameter is decreasing with time and levels off at the end of homogenization.

Example 4: Long-term stability of hard fat SLPs prepared by microfluidization.

The stability of hard fat SLPs is monitored over a period of one year. During this time the sample is stored in a refrigerator at approximately +4°C. After certain time intervals the particle size distribution of the sample is determined by PCS. Fig. 8 demonstrates that the mean particle size of hard fat SLPs is practically constant over the monitored period of one year.

Example 5: Preparation of unstable SLPs dispersions.

In a thermostated vial 4.0 g tripalmitate (Dynasan 116, Hüls AG) is heated to 75°C to melt the lipid. In the lipid melt 0.48 g soy bean lecithin (Lipoid S 100, Lipoid KG) is dispersed by probe sonication (MSE Soniprep 150) until the dispersion appears optically clear. 4 mg thiomersal and 0.9 g glycerol is dissolved in 35.6 ml bidistilled water. The aqueous phase is heated to 75°C and added to the lipid melt. A crude dispersion is produced by probe sonication for approximately 2 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 500 bar. The homogenized dispersion is allowed to stand at room temperature to cool off. On storage the SLP dispersion becomes a milky semi-solid, ointment-like gel.

3.0 g hard fat (Witepsol™ W35, Hüls AG) is melted in a thermostated vial at 75°C. 1.8 g soy bean lecithin (Phospholipon 100, Natterman) is dispersed in the tripalmitate melt as described in Example 1. The aqueous phase containing 10 mg thiomersal is heated to 75°C and added to the lipid melt to a weight of 100 g. A crude dispersion is produced by ultra-turrax vortexing for approximately 2 minutes. The crude dispersion is

transferred to a microfluidizer (Microfluidics Microfluidizer M-110T) which is immersed in a thermostated water bath (70°C). The dispersion is cycled through the microfluidizer for 10 minutes and allowed to stand at room temperature to cool off. On storage the SLP dispersion becomes a turbid semi-solid, ointment-like gel. A transmission electron micrograph of this gel is presented in Fig. 4.

In a thermostated vial 4.0 g tripalmitate (Dynasan 116, Hüls AG) is heated to 80°C to melt the lipid. In the lipid melt 0.8 g of a soy bean lecithin mixture (Lipoid S 75, Lipoid KG) is dispersed by probe sonication (MSE Soniprep 150) until the dispersion appears optically clear. 4 mg thiomersal is dissolved in 35.6 ml bidistilled water. The aqueous phase is heated to 80°C and added to the lipid melt. A crude dispersion is produced by probe sonication for approximately 2 minutes. The crude dispersion is transferred to a thermostated high pressure homogenizer (APV Gaulin Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 800 bar. The homogenized dispersion is filled in a glass vial and allowed to stand at room temperature to cool off. On cooling to room temperature the dispersion forms semi-solid looking fat aggregates on the wall of the glass vial. The dispersion gelatinizes when shear forces are applied, for example by passing it through a hypodermic syringe.

Obviously the use of phospholipids only as stabilizers, as found in commercial parenteral oil-in-water emulsions, does not yield stable systems in the case of SLP suspensions. Even the employment of phospholipids such as Lipoid S 75 which induces a considerably high negative net charge cannot provide a sufficient stabilization. Electrostatic repulsion alone cannot be the basic stabilization mechanism of SLPs as will be further outlined in Examples 6, 7 and 13.

Example 6: Preparation of tripalmitate SLPs sterically stabilized by tyloxapol.

A series of tripalmitate SLP dispersions stabilized by tyloxapol (Eastman Kodak) are prepared with varying lipid/surfactant ratios. The SLP dispersions are manufactured according to the following procedure:

Tyloxapol is dissolved in heated bidistilled water while the temperature is held below the cloud point of tyloxapol (approximately 90-95°C). The tyloxapol solution of a temperature of 80°C is added to the molten tripalmitate or, respectively, tripalmitate/lecithin dispersion of the same temperature. A crude emulsion is prepared by probe sonication for approximately 2 minutes. Then the crude emulsion is passed 5 times through a high-pressure homogenizer at a pressure of 1200 bar. The homogenized dispersion is allowed to stand at room temperature to cool off. All dispersions contain 2.5% glycerol and 0.01% thiomersal.

Table 1 gives the composition of the prepared SLP dispersions and their mean particle size after preparation (by number) as determined by PCS. The asterix (*) in the particle size column indicates that the dispersions display a bimodal size distribution with particle sizes considerably larger than indicated by the mean particle size. It turns out that sterically stabilized SLP dispersions require a high amount of surfactant in order to obtain homogeneously sized SLPs. In case of SLPs stabilized by tyloxapol and phospholipids the ratio of the surfactants needs to be optimized. In the present series a tyloxapol/lecithin ratio of at least 1:1 turned out to yield homogeneously sized SLPs. With increasing ratio the mean particle size is decreasing. As with examples 1 to 3 the addition of a highly mobile surfactant which is able to form micelles is required to obtain stable dispersions. The high amount of surfactant is needed to create a reservoir of surfactant in the dispersion medium that can provide enough surfactant molecules at the moment when the molten lipids recrystallize and form anisometrical particles with a large specific surface area.

Table 1: SLP dispersions sterically stabilized by tyloxapol.

Composition (w%)			Mean particle size (nm)
TP	Tyl	PL	
10	2	-	138.0*
10	4	-	84.9
10	0.7	2	487.4*
10	1	2	207.4*
10	2	2	102.8
10	4.5	3	60.9

Abbreviations: TP = tripalmitate, Tyl = tyloxapol, PL = phospholipids (Lipoid S 100).

Example 7: Preparation of tripalmitate SLPs sterically stabilized by poloxamers.

1.2 g soy bean lecithin (Lipoid S 100, Lipoid KG) is dispersed in 4.0 g molten tripalmitate (Dynasan 116, Hüls AG) by probe sonication at a temperature of 80°C. 1.8 g poloxamer (Pluronic™ F68, BASF), 0.9 g glycerol and 4 mg thiomersal is dissolved in 32.1 g bidistilled water heated to 80°C. The heated solution is added to the lipid melt and a crude dispersion is prepared by 2 minutes probe sonication. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 1200 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

The poloxamer stabilized SLPs display a monomodal size distribution with a mean particle size (by number) after preparation of 77.9 nm determined by PCS. Due to the presence of an excess of highly mobile surfactant in the aqueous phase the system is stabilized on recrystallization

of the molten lipids and a gelation as found with systems stabilized by phospholipids only does not occur.

5 Example 8: The influence of homogenization pressure on the mean particle size of SLPs.

SLPs of the following composition are prepared at different homogenization pressures. The SLP dispersions are composed of 3% tripalmitate (Dynasan 116, Hüls AG), 1.5% tyloxapol, 1% soy bean lecithin (Lipoid S 100, Lipoid KG), 0.01% thiomersal and bidistilled water to 100% (by weight). The lecithin is dispersed in the molten tripalmitate (80°C) by probe sonication until the dispersion appears optically clear. Tyloxapol is dissolved in warm water (80°C) containing thiomersal. The SLP dispersions are prepared as described in Example 6.

15 Fig. 9 displays the influence of homogenization pressure on the mean particle size of the SLPs. With increasing pressure the particle size is decreasing and the particle size distribution becomes more narrow.

Example 9: The influence of homogenization passes on the mean particle size of SLPs.

20 Tripalmitate SLPs composed of 3% tripalmitate (Dynasan 116, Hüls AG), 1.5% tyloxapol, 1% soy bean lecithin (Lipoid S 100, Lipoid KG), 0.01% thiomersal and bidistilled water to 100% (by weight) is prepared at a pressure of 800 bar as described in Example 6. Samples for size measurements are taken from the dispersion after preparation of the crude emulsion and after each pass through the homogenizer.

25 Fig. 10 presents the influence of the number of homogenization passes on the mean particle size of SLPs which is decreasing with increasing number of passes.

30 Example 10: Preparation of SLPs by probe sonication - Influence of sonication time on the mean particle size of SLPs.

In a sonication vial thermostated at 80°C 1.20 g tripalmitate is melted. In the lipid melt 0.40 g soy bean lecithin (Lipoid S 100) is dispersed

by probe sonication until the dispersion appears optically clear. 0.60 g tyloxapol and 4 mg thiomersal is dissolved in bidistilled water heated to 80°C. The aqueous phase is added to the lipid melt and an SLP dispersion is prepared by probe sonication at 80°C. The sonicator operates at 50% of its maximum power. At certain time intervals (1, 5, 10 and 15 minutes) samples are taken from the dispersion for size measurements. After 30 minutes probe sonication is stopped and the dispersion is allowed to stand at room temperature to cool off.

The influence of sonication time on the mean particle size of the SLPs is displayed in Fig. 11. With increasing sonication time the mean particle size is decreasing and the size distribution becomes more narrow.

Example 11: Preparation of SLPs by stirring.

An SLP dispersion composed as in Examples 9 and 10 is prepared by use of a heated magnetic stirrer (Pyro-Magnestir, Lab-Line). The lecithin is dispersed in the tripalmitate as described before. The heated aqueous phase is added to the melt. A dispersion is produced by stirring the mixture for 30 minutes at a temperature of 80°C. The dispersion is allowed to stand at room temperature to cool off.

The mean particle size after preparation (by volume) of the SLP dispersion is 59.5 µm determined by laser diffractometry (Malvern Mastersizer MS20). The maximum particle size measured is 250 µm. In contrast to high pressure homogenization and probe sonication, stirring produces relatively large particles in the micrometer size range.

Example 12: Influence of the matrix constituent on the mean particle size of SLPs.

SLP dispersions composed of 10% matrix constituent, 1.2% soy bean lecithin (Lipoid S 100), 0.4% sodium glycocholate, 2.25% glycerol and 0.01% thiomersal in bidistilled water to 100% are prepared as described in Example 1. Five different matrix constituents are employed: the waxes cetylpalmitate and white bees-wax and the triglycerides trilaurate, trimyristate and tripalmitate.

Table 2 presents the PCS mean particle sizes of the different SLP dispersions and the melting points of the matrix constituents.

Table 2: Influence of matrix constituents.

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Matrix	Melting point (°C)	Mean size of SLPs (nm)
Cetylpalmitate	45.5	141.0
White bees-wax	62.5	195.3
Trilaurate	45.0	137.2
Trimyristate	56.5	161.1
Tripalmitate	63.0	209.2

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The mean particle size of SLPs is increasing with the melting point of the matrix constituent.

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Example 13: Influence of emulsifier type and amount on the mean particle size and stability of SLPs.

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Tripalmitate SLP dispersions with different types and amounts of emulsifiers are prepared as described in Example 2. The composition of the different batches is given in Table 3. All dispersions contain 2.25% glycerol and 0.01% thiomersal.

The mean particle size of the different batches of SLPs is presented in Fig. 12. The mean particle size depends on the type and amount of emulsifier.

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Table 3: Compositions of SLP batches (in w%).

Batch no	TP	PL	SGC	Plu
1	10%	1.2%	-	-
2	10%	1.2%	0.4%	-
3	10%	2.4%	0.4%	-
4	10%	-	-	1.8%
5	10%	-	-	3.6%

Abbreviations: TP = tripalmitate, PL = phospholipids (Lipoid S 100), SGC = sodium glycocholate, Plu = Pluronic F68.

The combination of phospholipids and bile salts is most efficient with regard to the mean particle size and the stability. The system stabilized by phospholipids only gelatinizes and forms an ointment-like semi-solid gel on storage. The systems stabilized by Pluronic F68 tend to gelatinize when shear forces are applied, i.e. when the particles are forced to get closer to each other. Obviously the steric stabilization by poloxamers is not sufficient in this case. As a result the optimum stabilization is that by a surfactant combination of emulsifiers that are present in and act from the lipid side (such as phospholipids) and of emulsifiers that constitute a reservoir of highly mobile surfactant molecules in the dispersion medium (such as bile salts, tyloxapol and poloxamers). Though repulsion forces represent an important factor for the long-term stability, the basic mechanism of SLP stabilization is the high mobility of the excess of surfactant which provides for the immediate surface coverage of newly created surfaces during recrystallization of the molten lipids.

Example 14: Effect of bile salt as co-emulsifier of phospholipid stabilized SLPs.

Phospholipid-stabilized SLP dispersions employing different matrices (tripalmitate, hard fat) are prepared with or without the addition of bile salt (sodium glycocholate) to the aqueous phase according to the method described in Example 1. All dispersions contain 2.25% glycerol and 0.01% thiomersal. Emulsification of the crude dispersions is performed by high pressure homogenization (APV Gaulin Micron Lab 40) under different homogenization conditions. The following dispersions were prepared:

Composition	Homogenization conditions
7.0 g TP, 0.84 g PL, 62.2 g H ₂ O	3 x 500 bar
7.0 g TP, 0.84 g PL, 0.28 g BS, 61.9 g H ₂ O	3 x 500 bar
7.0 g TP, 0.84 g PL, 62.2 g H ₂ O	10 x 1200 bar
7.0 g TP, 0.84 g PL, 0.28 g BS, 61.9 g H ₂ O	10 x 1200 bar
7.0 g HF, 0.84 g PL, 62.2 g H ₂ O	3 x 500 bar
7.0 g HF, 0.84 g PL, 0.28 g BS, 61.9 g H ₂ O	3 x 500 bar
7.0 g HF, 0.84 g PL, 62.2 g H ₂ O	10 x 1200 bar
7.0 g HF, 0.84 g PL, 0.28 g BS, 61.9 g H ₂ O	10 x 1200 bar

Abbreviations: BS = bile salt; H₂O = bidistilled water, HF = hard fat (Witepsol W35); PL = phospholipids (Lipoid S 100); TP = tripalmitate.

The mean particle size of the dispersions as determined by PCS after preparation is presented in Fig. 13. This example demonstrates the effect of bile salts as co-emulsifier in the aqueous phase on the particle size of phospholipid stabilized SLPs. It is clearly shown that the addition of bile salts reduces the mean particle size of SLPs by up to 57%. Thus, by the use of bile salts as co-emulsifier extremely fine dispersions can be obtained. The effect of the bile salt can be attributed to the high mobility of this micelle forming ionic surfactant which enables the surfactant molecules to immediately cover freshly generated surfaces during the homogenization process. The phospholipids which tend to form liquid crystalline structures in

the aqueous phase are not sufficiently mobile to provide the immediate stabilization of freshly created particles so that instantaneous coalescence occurs in case there is no highly mobile co-surfactant in the aqueous phase.

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Example 15: Preparation of trimyristate SLPs stabilized by a lecithin/bile salt blend.

In a thermostated vial 7.0 g trimyristate (Dynasan 114, Hüls AG) is melted at 70°C. 0.96 g phospholipids (Lipoid S 100) are dispersed in the melt by probe sonication. A solution of 280 mg sodium glycocholate, 1.6 g glycerol and 7 mg thiomersal in 61 ml bidistilled water is heated to 70°C and added to the melt. A crude dispersion is prepared by probe sonication for approximately 2 minutes. The crude dispersion is transferred to a high pressure homogenizer (APV Gaulin Micron Lab 40) thermostated at approximately 90°C and is passed 5 times through the homogenizer at a pressure of 500 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

The mean particle size after preparation determined by PCS is 155.7 nm. In laser diffractometry (Malvern Mastersizer MS20) no particles above 1 µm can be detected. The particle size distribution derived from laser diffractometry is presented in Fig. 14. This example demonstrates that the use of bile salts as co-emulsifier of phospholipid stabilized SLPs efficiently prevents the formation of particles larger than 1 µm due to the rapid coverage of freshly created surfaces during homogenization, thereby minimizing immediate coalescence.

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Example 16: Preparation of tripalmitate SLPs without ultrasonication (method A).

In a thermostated vial 4.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 85°C. 0.96 g lecithin (Lipoid S 100) is dissolved in ethanol. The lecithin solution is added to the melt. The ethanol is evaporated at a temperature of 85°C. 160 mg sodium glycocholate, 0.9 g glycerol and 4 mg thiomersal is dissolved in 35 ml bidistilled water. The solution is heated to

30

85°C and added to the melt. A crude dispersion is prepared by ultra-turrax vortexing for approximately 2 minutes. The crude dispersion is transferred to a high-pressure homogenizer (APV Gaulin Micron Lab 40) thermostated at approximately 90°C and passed 10 times through the homogenizer at a pressure of 800 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

Example 17: Preparation of tripalmitate SLPs without ultrasonication (method B).

In a thermostated vial 4.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 85°C. 0.96 g lecithin (Lipoid S 100) is added to the melt. The mixture is shaken until the lecithin is completely dispersed in the melt and the dispersion appears isotropic. 160 mg sodium glycocholate, 0.9 g glycerol and 4 mg thiomersal is dissolved in 35 ml bidistilled water. The solution is heated to 85°C and is added to the melt. A crude dispersion is prepared by ultra-turrax vortexing for approximately 2 minutes. The crude dispersion is transferred to a high-pressure homogenizer (APV Gaulin Micron Lab 40) thermostated at approximately 90°C and passed 10 times through the homogenizer at a pressure of 800 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

Example 18: Preparation of tripalmitate SLPs by dispersing phospholipids in the aqueous phase.

In a thermostated vial 4.0 g tripalmitate (Dynasan 116) is melted at 80°C. 0.96 g phospholipids (Lipoid S 100) is dispersed in 35 ml of an aqueous solution of 160 mg sodium glycocholate, 0.9 g glycerol and 4 mg thiomersal by stirring for approximately one hour. The phospholipid dispersion is heated to 80°C and added to the tripalmitate melt. A crude dispersion is prepared by probe sonication for approximately 2 minutes. The crude dispersion is transferred to a high-pressure homogenizer (APV Gaulin Micron Lab 40) thermostated at approximately 90°C and passed 10 times through the homogenizer at a pressure of 800 bar. The homogenized dispersion is allowed to stand at room temperature to cool off.

Example 19: Preparation of tripalmitate SLPs stabilized by a highly mobile surfactant.

In a thermostated vial 5.0 g tripalmitate is melted at 80°C. 600 mg sodium glycocholate is dissolved in 44.4 g bidistilled water containing 1.13 g glycerol and 0.01% thiomersal. The aqueous solution is heated to 80°C and added to the melt. A crude dispersion is prepared by sonication for approximately 5 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 8 times through the homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at room temperature to cool off.

The mean particle size (by number) of the SLP dispersion after preparation is 96.8 nm determined by PCS. The size distribution is narrow and monomodal.

This example demonstrates that it is possible to prepare small homogeneously sized SLPs by the use of one surfactant only, such as the bile salt sodium glycocholate, provided the surfactant is highly mobile and constitutes a reservoir of stabilizer in the aqueous phase in order to provide for the stabilization of newly created surfaces during recrystallization of the SLP matrix.

Example 20: Long-term stability of different SLP dispersions.

Several different SLP dispersions are prepared according to the method described in Example 2. All dispersions contain 2.25 % glycerol as isotonicity agent and 0.01 % thiomersal as a preservative. The long-term stability of the dispersions is judged from repeated size measurements (by PCS) over a period of 18 months. The dispersions are stored at refrigeration temperatures. For comparison a soy bean oil emulsion system is included. The composition of the dispersions and their mean particle sizes during storage are summarized in Table 4.

Table 4

	Composition (w %)			Mean particle size (nm) after	
	Matrix	PI	SGC	Preparation	18 months
5	10% TP	1.2%	0.4%	125.9	121.6
	10% TP	2.4%	0.4%	104.5	111.2
	10% TP	2.4%	0.4%	103.6	104.7
	9.5% TP ¹⁾				
	0.5% GMS	2.4%	0.4%	102.4	102.4
10	10% SO	2.4%	0.4%	129.6	139.9

Abbreviations: PL = phospholipid (Lipoid S 100); SGC = sodium glyco-
cholate; TP = tripalmitate; GMS = glycerol monostearate; SO = soy bean
oil.

- 15 ¹⁾ The SLP dispersion contains 5 % (related to fat phase) of the cardio-
protective drug ubidecarenone.

It is shown that the mean particle size of the dispersions remains
practically unchanged during storage for 18 months. Thus, the results
20 demonstrate that drug-free and drug-loaded SLP dispersions exhibit a long-
term stability similar to that of lipid emulsions.

Example 21: Sterile filtration of tripalmitate SLPs.

40 ml of a crude SLP dispersion composed of 3% tripalmitate
25 (Dynasan 116, Hüls AG), 1.5% tyloxapol, 1% lecithin (Lipoid S 100), 2.25%
glycerol and 0.01% thiomersal in bidistilled water to 100% is prepared
according to the method described in Example 6. The crude dispersion is
passed 5 times through a thermostated homogenizer (APV Micron Lab 40)
at a pressure of 1200 bar. Half the volume of the batch is allowed to stand
30 at room temperature to cool off, whereas the rest is filtered through a sterile

syringe filter (Nalgene SFCA, 0.2 μm pore size) before being cooled to the recrystallization temperature of the molten lipids.

The particle size distribution of both samples is determined by PCS. The mean particle size of the unfiltered sample is 56.7 nm and that of the sterile filtered SLP dispersion is 53.2 nm, i.e. both samples have practically the same mean particle size.

Example 22: Sterilization of tripalmitate SLPs by autoclaving.

40 ml of a crude SLP dispersion composed of 3% tripalmitate (Dynasan 116, Hüls AG), 1.8% lecithin (Lipoid S 100), 0.6% sodium glycolate, 2.25% glycerol and 0.01% thiomersal in bidistilled water to 100% is prepared according to the method described in Example 2. The crude dispersion is passed 10 times through a thermostated homogenizer (APV Micron Lab 40) at a pressure of 1200 bar.

Before being cooled to the recrystallization temperature of the molten lipids the SLP dispersion is filled into an injection vial and sterilized by autoclaving at 121°C/2 atm for 45 minutes. The autoclaved dispersion is allowed to stand at room temperature to cool off. It shows no signs of aggregation or phase separation and has a mean particle size of 65.9 nm determined by PCS.

Example 23: Lyophilization of SLPs.

In a thermostated vial 3.5 g tripalmitate (Dynasan 116, Hüls AG) is melted at 75°C, and 1.05 g lecithin (Lipoid S 100, Lipoid KG) is dispersed in the melt by probe sonication. 1.58 g tyloxapol, 14 g sucrose and 7 mg thiomersal is dissolved in 50 ml distilled water heated to 75°C and the aqueous phase is added to the lipid melt. A crude dispersion is prepared by probe sonication and then passed 5 times through a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) at a pressure of 900 bar. The homogenized dispersion is passed through a 0.2 μm sterile filter.

For lyophilization the dispersion is filled into plane-bottom vials which are immersed in liquid nitrogen for 1 minute and transferred to the freeze-

drying chamber. Samples are freeze-dried for 36 hours under vacuum at a recipient temperature of -40°C.

Freeze-drying yields easily redispersible fine powders. The particle size of the SLP dispersions is determined by PCS prior to lyophilization and after reconstitution of the lyophilized powders with distilled water. The mean particle size prior to lyophilization is 79 nm and that of the reconstituted dispersion is 87 nm, i.e. there is practically no change in mean particle size after lyophilization.

10 Example 24: Surface modification by adsorption of polymers.

In a thermostalized vial 4.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 80°C and 1.6 g soy bean lecithin (Lipoid S 100) is dispersed in the melt by probe sonication. 35.25 g of an aqueous solution of 0.01% thiomersal is heated to 80°C and added to the melt. A crude dispersion is produced by probe sonication which is then passed five times through a high-pressure homogenizer at a pressure of 1200 bar. The dispersion is filtered through a 0.2 µm syringe filter. The batch is divided into 3 parts of equal volumes. One part is diluted with the same amount of water and stored at 90°C to prevent gelation of the phospholipid stabilized dispersion on cooling down below the recrystallization temperature. The other two parts of the batch are incubated overnight with equal volumes of 6% (w/w) poloxamer 407 (Pluronic F127, BASF) and 6 % (w/w) poloxamine 908 (Tetronic 908, BASF) solution, respectively, in such a way that the polymer solution is added to the SLP dispersion prior to being cooled below the recrystallization temperature of SLPs to provide for the immediate availability of polymer molecules as soon as new surfaces are created due to recrystallization. Both polymers have been described in literature to modify the bio-distribution of intravenously administered colloidal particles.

The modification of the surface properties of tripalmitate SLPs is demonstrated by differences in the zeta potential. Zeta potentials were determined by laser Doppler anemometry in a microelectrophoresis cell (Malvern Zetasizer 3). The results are summarized in Table 5.

Table 5: Zetapotential of surface modified SLPs.

Composition [% (w/w)]			Zetapotential [mV]
TP	PL	Polymer	
5%	2%	-	-29.6
5%	2%	3% F127	-1.9
5%	2%	3% T908	-2.9

Abbreviations: TP = tripalmitate, PL = phospholipids, F127 = pluronic F127, T908 = Tetronic 908.

The incubation of SLPs with block copolymers of the poloxamer and poloxamine type results in a decrease of the zetapotential. Due to the adsorption of the polymers the surfaces become more hydrophobic. The hydrophobicity of the surface is described to be one of the factors governing the RES (reticuloendothelial system) activity and the biodistribution of colloidal particles.

Example 25: Preparation of SLPs loaded with the cardio-protective drug ubidecarenone.

Three different types of SLPs containing the cardio-protective drug ubidecarenone are prepared. The SLPs are composed as summarized in table 6. All dispersions contain 2.25% glycerol and 0.01% thiomersal.

Batch 1 and 2 are prepared by dispersing lecithin in the molten matrix constituent as described before. In this melt ubidecarenone is dissolved. After addition of the aqueous phase containing sodium glycolate, glycerol and thiomersal a crude dispersion is prepared by probe sonication. It is transferred to a thermostated homogenizer (APV Micron Lab 40) and passed through the homogenizer ten times at a pressure of 800 bar. The dispersions are allowed to stand at room temperature to cool off.

Batch 3 is prepared by dispersing lecithin in the molten matrix constituent as described before. In this melt ubidecarenone is dissolved. After addition of the aqueous phase containing tyloxapol, glycerol and thiomersal, a crude dispersion is prepared by probe sonication. It is transferred to a thermostated homogenizer (APV Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 1200 bar. The dispersion is allowed to stand at room temperature to cool off.

Table 6: Ubidecarenone-loaded SLPs.

Batch no	Composition	Mean particle size
1	10% TP, 2.4% PL, 0.4% SGC, 1% Ubi	80.2 nm
2	10% HF, 1.2% PL, 0.4% SGC, 1% Ubi	78.9 nm
3	3% TP, 1.5% Tyl, 1% PL, 0.2% Ubi	46.8 nm

Abbreviations: TP = tripalmitate, PL = phospholipids, SGC = sodium glycocholate, Ubi = Ubidecarenone, HF = hard fat (Witepsol W35), Tyl = Tyloxapol.

Example 26: Preparation of SLPs loaded with oxazepam.

In a thermostated vial 7.0 g tripalmitate is melted at 80°C. 1.68 g lecithin and 140 mg oxazepam is dispersed in the melt by probe sonication. 60 ml of heated aqueous phase containing 280 mg sodium glycocholate, 1.58 g glycerol and 7 mg thiomersal is added to the melt and a crude dispersion is prepared by probe sonication. The crude dispersion is homogenized by passing 10 times through a thermostated high-pressure homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at room temperature to cool off. The dispersion of oxazepam loaded SLPs has a mean particle size of 122.7 nm after preparation.

Example 27: Preparation and long-term stability of SLPs loaded with diazepam.

In a thermostated vial 4.0 g tripalmitate is melted at 80°C. 0.96 g lecithin and 120 mg diazepam is dispersed in the melt by probe sonication. 35 ml of heated aqueous phase containing 160 mg sodium glycocholate, 0.9 g glycerol and 4 mg thiomersal is added to the melt and a crude dispersion is prepared by probe sonication. The crude dispersion is homogenized by passing 10 times through a thermostated high-pressure homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at room temperature to cool off.

The dispersion of diazepam-loaded SLPs has a mean particle size after preparation of 104.6 nm. After 12 months of storage the mean particle size determined by PCS is 113.9 nm. Precipitation of drug substance during storage is not detected macroscopically. Investigations of the dispersion by polarized light microscopy over the monitored period of 12 months do not reveal the presence of drug crystals.

Example 28: Preparation of SLPs loaded with lidocaine.

In a thermostated vial 4.0 g tripalmitate is melted at 80°C. 0.96 g lecithin and 60 mg lidocaine is dispersed in the melt by probe sonication. 35 ml of heated aqueous phase containing 320 mg sodium glycocholate, 0.9 g glycerol and 4 mg thiomersal is added to the melt and a crude dispersion is prepared by probe sonication. The crude dispersion is homogenized by passing 10 times through a thermostated high-pressure homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at room temperature to cool off.

The dispersion of lidocaine loaded SLPs has a mean particle size after preparation of 90.4 nm.

Example 29: Preparation and long-term stability of SLPs loaded with prednisolone.

In a thermostated vial 4.0 g tripalmitate is melted at 80°C. 0.48 g lecithin and 80 mg prednisolone is dispersed in the melt by probe sonica-

tion. 36 ml of heated aqueous phase containing 160 mg sodium glyco-
cholate, 0.9 g glycerol and 4 mg thiomersal is added to the melt and a
crude dispersion is prepared by probe sonication. The crude dispersion is
homogenized by passing 10 times through a thermostated high-pressure
5 homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at
room temperature to cool off.

The dispersion of prednisolone-loaded SLPs has a mean particle size
after preparation of 118.3 nm. After 12 months of storage the mean particle
size determined by PCS is 124.2 nm. Precipitation of drug substance during
10 storage is not detected macroscopically. Investigations of the dispersion by
polarized light microscopy over the monitored period of 12 months do not
reveal the presence of drug crystals.

Example 30: Preparation of SLPs loaded with cortisone.

15 Four different types of SLPs containing cortisone are prepared. The
SLPs are composed as summarized in table 7. All dispersions contain
2.25% glycerol and 0.01 thiomersal.

Batch 1 and 2 are prepared by dispersing lecithin in the molten
matrix constituent as described before. In this melt cortisone is dissolved.
20 After addition of the aqueous phase containing sodium glycocholate, glyce-
rol and thiomersal, a crude dispersion is prepared by probe sonication. It is
transferred to a thermostated homogenizer (APV Micron Lab 40) and
passed 10 times through the homogenizer. The dispersions are allowed to
stand at room temperature to cool off.

25 Batch 3 is prepared by dispersing lecithin in the molten matrix
constituent as described before. In this melt cortisone is dissolved. After
addition of the aqueous phase containing poloxamer (Pluronic F68), glycerol
and thiomersal, a crude dispersion is prepared by probe sonication. It is
transferred to a thermostated homogenizer (APV Micron Lab 40) and
30 passed 5 times through the homogenizer at a pressure of 1200 bar. The
dispersion is allowed to stand at room temperature to cool off.

Batch 4 is prepared by dispersing lecithin in the molten matrix con-
stituent as described before. In this melt cortisone is dissolved. After addi-

tion of the aqueous phase containing tyloxapol, glycerol and thiomersal a crude dispersion is prepared by probe sonication. It is transferred to a thermostated homogenizer (APV Micron Lab 40) and passed through the homogenizer five times at a pressure of 1200 bar. The dispersion is allowed to stand at room temperature to cool off.

Table 7: Cortisone-loaded tripalmitate SLPs.

Batch no	Composition	Mean particle size
1	10% TP, 1.2% PL, 0.4% SGC, 0.2% Cort	124.2 nm
2	3% TP, 1.8% PL, 0.6% SGC, 0.3% Cort	67.3 nm
3	10% TP, 4.5% Plu, 3% PL, 0.1% Cort	70.5 nm
4	3% TP, 1.5% Tyl, 1% PL, 0.1% Cort	48.5 nm

Abbreviations: TP = tripalmitate, PL = phospholipids, SGC = sodium glycocholate, Cort = Cortisone, Plu = Pluronic F68, Tyl = Tyloxapol.

Example 31: Tripalmitate SLPs loaded with retinol (vitamin A).

In a thermostated vial 1.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 80°C. 60 mg retinol (vitamin A-alcohol >99 %, Fluka) is dissolved in the melt. 300 mg soy bean lecithin (Lipoid S 100) is dispersed in the melt by probe sonication until the dispersion appears optically clear. 450 mg poloxamer 407 (Pluronic™ F127, BASF) is dissolved in 29.0 g bidistilled water. The aqueous phase is heated to 80°C and added to the melt. A fine dispersion is prepared by probe sonication for 20 minutes. The dispersion is filtered through a 0.2 µm syringe filter to remove metal shed from the ultrasound probe. The dispersion is allowed to stand at room temperature to cool off.

The mean particle size by number after preparation of vitamin A-loaded tripalmitate SLPs is 98.5 nm determined by PCS.

Example 32: Tripalmitate SLPs loaded with phytylmenadione (vitamin K₃).

In a thermostated vial 1.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 80°C. 60 mg phytylmenadione (vitamin K₃, Sigma) and 300 mg soy bean lecithin (Lipoid S 100) is dispersed in the melt by probe sonication until the dispersion appears optically clear. 450 mg poloxamer 407 (Pluronic™ F127, BASF) is dissolved in 28.7 g bidistilled water. The aqueous phase is heated to 80°C and added to the melt. A fine dispersion is prepared by probe sonication for 20 minutes. The dispersion is filtered through a 0.2 µm syringe filter to remove metal shed from the ultrasound probe. The dispersion is allowed to stand at room temperature to cool off. The mean particle size by number after preparation of vitamin K₃-loaded tripalmitate SLPs is 86.8 nm determined by PCS.

Example 33: Preparation of tripalmitate SLPs loaded with estramustine.

In a thermostated vial 7.0 g tripalmitate (Dynasan 116, Hüls AG) is melted at 80°C. In the melt 1.68 g soy bean lecithin (Lipoid S 100) is dispersed by probe sonication until the dispersion appears optically clear. 40 mg estramustine is dissolved in the tripalmitate/lecithin dispersion. 0.42 g sodium glycocholate and 1.58 g glycerol is dissolved in 60 g bidistilled water. The aqueous phase is heated to 80°C and added to the melt. A crude emulsion is prepared by probe sonication for approximately 2 minutes. The crude emulsion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 10 times through the homogenizer at a pressure of 800 bar. The dispersion is allowed to stand at room temperature to cool off.

Example 34: Physical state of different SLPs at body temperature.

Two batches of SLPs from different matrix constituents are prepared according to the method described in Example 2. Batch 1 is composed of 10% tripalmitate, 0.5% ubidecarenone, 1.2% soy bean lecithin (Lipoid S 100, Lipoid KG), 0.4% sodium glycocholate, 2.25% glycerol, 0.01% thiomersal and bidistilled water to 100% (by weight). Batch 2 is composed of 10% hard fat (Witepsol™ W35, Hüls AG), 0.5% ubidecarenone, 1.2% soy

bean lecithin (Lipoid S 100, Lipoid KG), 0.4% sodium glycocholate, 2.25% glycerol, 0.01% thiomersal and bidistilled water to 100% (by weight).

The physical state of the matrix constituents is determined by synchrotron radiation X-ray diffraction at 20°C and at 38°C. The samples are placed in thermostated sample holders. The diffraction patterns are recorded for 180 seconds each. Fig. 15a demonstrates that at room temperature (20°C) both batches of SLPs are crystalline. The spacings correspond to the α -crystalline polymorphs. At body temperature (38°C) the tri-palmitate SLPs are still crystalline whereas no reflections can be detected for the hard fat SLPs, i.e. they are amorphous and molten (Fig. 15b). The different physical states of these SLPs at body temperature give rise to a different biopharmaceutical behaviour with respect to the release of incorporated drugs or bioactive agents. SLPs molten at body temperature display basically the release characteristics typical of conventional lipid emulsions. Due to the free diffusion of drug molecules in the liquid lipid the drug can be released from the vehicle relatively fast. In contrast, SLPs which are solid at body temperature give rise to sustained release of incorporated drugs. Since the drug molecules are immobilized in the solid matrix drug release is not diffusion-controlled but depends on the degradation of the solid lipid matrix in the body and is therefore delayed.

Example 35: Preparation of PBAs from miconazole.

In a thermostated vial 0.4 g miconazole is melted at 90°C. 0.24 g lecithin (Lipoid S 100) is dispersed in the melt by probe sonication until the dispersion appears optically clear. 0.9 g glycerol, 80 mg sodium glycocholate and 4 mg thiomersal is dissolved in 38.5 ml bidistilled water and heated to 90°C. The aqueous phase is added to the miconazole/lecithin melt and a crude dispersion is produced by probe sonication for 5 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 10 times through the homogenizer at a pressure of 800 bar. The PBA dispersion is allowed to stand at room temperature to cool off.

On cooling the molten miconazole recrystallizes and forms a suspension of miconazole microparticles. The mean particle size (by volume) of miconazole PBAs is 21.8 μm determined by laser diffractometry. The sediment of miconazole PBAs is easily redispersible by slight agitation.

5

Example 36: Preparation of PBAs from ibuprofen.

In a thermostated vial 1.2 g ibuprofen is melted at 85°C. 0.72 g lecithin (Lipoid S 100) is dispersed in the melt by probe sonication until the dispersion appears optically clear. 0.9 g glycerol, 240 mg sodium glycocholate and 4 mg thiomersal is dissolved in 37 ml bidistilled water and heated to 85°C. The aqueous phase is added to the ibuprofen/lecithin melt and a crude dispersion is produced by probe sonication for 5 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 6 times through the homogenizer at a pressure of 800 bar. The PBA dispersion is allowed to stand at room temperature to cool off.

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On cooling the molten ibuprofen recrystallizes and forms a suspension of ibuprofen microparticles. The mean particle size (by volume) of ibuprofen PBAs is 61.4 μm determined by laser diffractometry. The sediment of ibuprofen PBAs is easily redispersible by slight agitation.

20

Example 37: Dissolution speed of ibuprofen PBAs.

The dissolution speed of ibuprofen PBAs of Example 36 is measured in a laser diffractometer (Malvern Mastersizer MS20) by monitoring the decay of the so-called obscuration over a period of 10 minutes. The obscuration is a measure of the reduced intensity of unscattered laser light by a sample and is related to the concentration of particles in the laser beam. In parallel the particle size can be measured. For measurement the ibuprofen PBA sample is diluted with water and dispersed by magnetic stirring in a measuring cell placed in the laser beam line. Fig. 16 presents the decay of obscuration and particle size of a sample of ibuprofen PBAs. Within 10 minutes the obscuration has decayed to zero, i.e. there is no detectable amount of particles hinting at the complete dissolution of the PBAs. The

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dissolution of the untreated raw substance ibuprofen cannot be measured by this technique since the substance is only poorly wettable in water.

Example 38: Preparation of PBAs from lidocaine.

5 In a thermostated vial 1.2 g lidocaine is melted at 80°C. 1.2 g tyloxapol is dissolved in 37.6 ml bidistilled water and heated to 80°C. The aqueous phase is added to the lidocaine melt and a crude dispersion is produced by probe sonication for 2 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron
10 Lab 40) and passed 5 times through the homogenizer at a pressure of 1200 bar. The PBA dispersion is allowed to stand at room temperature to cool off.

On cooling the molten lidocaine recrystallizes into fine needles and forms a suspension of lidocaine microparticles. Fig. 17 shows a polarized
15 microscopic picture of the suspended lidocaine needles. The particle shape of the raw material lidocaine-base (Synopharm) is different from that of lidocaine PBAs as demonstrated by the polarized microscopic picture of Fig. 18.

The mean particle size (by volume) of lidocaine PBAs is 174.2 µm
20 determined by laser diffractometry. The maximum detected particle size is 400 µm. The sediment of lidocaine PBAs is easily redispersible by slight agitation. The addition of water to PBAs leads to the rapid dissolution of the particles. In contrast the raw material lidocaine is only sparingly soluble in water and the dissolution speed is much slower. The high dissolution speed
25 of lidocaine PBAs is a consequence of the modified surface properties and the finely dispersed state of the particles. Due to the rapid dissolution a determination of the dissolution speed according to the method described in Example 37 is not possible.

30 Example 39: Preparation of PBAs from cholecalciferol (vitamin D₃).

In a thermostated vial 0.8 g cholecalciferol is melted at 95°C. 120 mg soy bean lecithin (Lipoid S 100) is dispersed in the melt by probe sonication until the dispersion appears optically clear. 40 mg sodium glycochol-

ate and 0.9 g glycerol is dissolved in 37.92 ml bidistilled water and heated to 95°C. The aqueous phase is added to the cholecalciferol/lecithin dispersion and a crude dispersion is produced by probe sonication for 5 minutes. The crude dispersion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 8 times through the homogenizer at a pressure of 1200 bar. The PBA dispersion is allowed to stand at room temperature to cool off.

The mean particle size after preparation by number of cholecalciferol PBAs is 325.1 nm determined by PCS.

Example 40: Preparation of PBAs from estramustine.

In a thermostated vial 2 g estramustine is melted at 105°C. In the melt 0.8 g soy bean lecithin (Lipoid S 100) is dispersed by probe sonication until the dispersion appears optically clear. 0.2 g sodium glycocholate and 0.9 g glycerol is dissolved in 36.1 g bidistilled water. The aqueous phase is heated to 95°C and added to the melt. A crude emulsion is prepared by probe sonication for approximately 5 minutes. The crude emulsion is transferred to a thermostated high-pressure homogenizer (APV Gaulin Micron Lab 40) and passed 5 times through the homogenizer at a pressure of 1200 bar. The dispersion is allowed to stand at room temperature to cool off.

LEGENDS OF FIGURES

- Fig. 1 Transmission electron micrograph of tripalmitate SLPs of Example 1 after 5 months of storage at room temperature. The bar represents 400 nm.
- Fig. 2 Differential scanning calorimetric (DSC) thermogram of a) pure tripalmitate and of b) tripalmitate SLPs of Example 1. The transition peaks correspond to the melting of the β -crystalline polymorph.
- Fig. 3 Synchrotron radiation wide angle X-ray diffraction pattern of tripalmitate SLPs of Example 2. The reflexions correspond to the β -crystalline polymorph.
- Fig. 4 Transmission electron micrograph of unstable hard fat SLPs of Example 5. The SLP dispersion gelatinized on storage by forming a three-dimensional network. The bar corresponds to 1000 nm.
- Fig. 5 Particle size distribution of tripalmitate SLPs of Example 1 after 15 months of storage. The graph represents the result of a multiangle PCS measurement.
- Fig. 6 PCS particle size distribution of a 10% tripalmitate SLP dispersion compared to that of the commercial lipid emulsion Intralipid® 10%.
- Fig. 7 Influence of microfluidization time on the mean particle size of hard fat SLPs of Example 3.
- Fig. 8 Stability on storage of hard fat SLPs of Example 3 as indicated by the development of the mean particle size with storage time (monitored period: 12 months).
- Fig. 9 Influence of homogenization pressure on the mean particle size of tripalmitate SLPs.
- Fig. 10 Influence of the number of homogenization passes on the mean particle size of tripalmitate SLPs (no 0 corresponds to the crude dispersion prepared by sonication).

- Fig. 11 Influence of sonication time on the mean particle size of tri-palmitate SLPs prepared by probe sonication.
- Fig. 12 Influence of type and amount of emulsifier on the mean particle size of tripalmitate SLPs prepared according to Example 13.
- 5 Fig. 13 Effect of bile salts as co-emulsifier on the mean particle size of different phospholipid stabilized SLP dispersions of Example 14.
- Fig. 14 Particle size distribution of trimyrystate SLPs of Example 15 as determined by laser diffractometry.
- 10 Fig. 15 The physical state of different drug-loaded SLPs a) at 20°C and b) at 38°C determined by synchrotron radiation wide-angle X-ray diffraction.
- Fig. 16 Dissolution speed of ibuprofen PBAs of Example 37.
- 15 Fig. 17 Polarized microscopic picture of lidocaine PBAs of Example 38 (magnification: 150 x).
- Fig. 18 Polarized microscopic picture of lidocaine raw material employed for the production of lidocaine PBAs (magnification: 150 x).

CLAIMS

1. A process for emulsifying insoluble or sparingly water-soluble agents which are solid at room temperature, c h a r a c t e r i z e d in that the following steps are carried out:
- 5
- a. the solid agent or a mixture of solid agents is melted,
- b. a dispersion medium is heated to approximately the same temperature as the molten solid agent or the mixture of solid agents,
- 10
- c. one or more highly mobile water-soluble or dispersible stabilizer/stabilizers which does/do not form a separate phase in the dispersion medium is/are added to the dispersion medium in such a way that the amount of highly mobile stabilizers is, after emulsification, sufficient to stabilize newly created surfaces during recrystallization. Optionally, one or more lipid-soluble or -dispersible stabilizers is/are added additionally to the molten agent or mixture of agents,
- 15
- d. the molten agent or mixture of agents and the dispersion medium is/are emulsified in a liquid phase by high-pressure homogenization, sonication, high-speed stirring, vortexing and/or hand shaking, preferably by high-pressure homogenization,
- 20
- e. the homogenized dispersion is allowed to cool until solid particles are formed by recrystallization of the dispersed agents.
- 25
2. A process according to claim 1, c h a r a c t e r i z e d in that the homogenized dispersion is passed through a filter prior to cooling below the recrystallization temperature to remove particulate contaminations in such a way that the filter pore size is chosen large enough not to retain the particles of emulsified, molten agents.
- 30
3. A process according to claim 1 or 2, c h a r a c t e r i z e d in that the agent or mixture of agents is/are a lipid/lipids preferably having melting points between approximately 30°C and 120°C, preferably

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constituted of mono-, di- and triglycerides of long chain fatty acids; hydrogenated vegetable oils; fatty acids and their esters; fatty alcohols and their esters and ethers; natural or synthetic waxes such as beeswax and carnauba wax; wax alcohols and their esters, sterols such as cholesterol and its esters, hard paraffins or mixtures of the above mentioned lipids.

4. A process according to claim 1 or 2, characterized in that the agent or mixture of agents is/are a bioactive agent/bioactive agents or drug/drugs preferably showing a low bioavailability and/or being badly absorbed from the intestinum, having melting points preferably below 100°C or the melting points of which can be decreased to below 100°C by addition of adjuvants, preferably anesthetics and narcotics such as butanilicaine, fomocaine, isobutambene, lidocaine, risocaine, pseudococaine, prilocaine, tetracaine, trimecaine, tropacocaine, and etomidate; anticholinergics such as metixen and profenamine; antidepressives, psychostimulants and neuroleptics such as alimenazine, binedaline, perazine, chlorpromazine, fenpenta-
diol, fenanisol, mebenazine, methylphenidate, thioridazine, toloxaton and trimipramide; antiepileptics such as dimethadion and nicethamide; antimycotics such as butoconazole, chlorphenesin, etisazole, exalamid, precilocine and miconazole; antiphlogistics such as butibufen and ibuprofen; bronchodilators such as bamifylline; cardiovascular drugs such as alprenolol, butobendine, clordiazole, hexobendine, nicofibrate, penbutolol, pirmenol, prenylamine, procaine amide, propatrylnitrate, suloctidil, toliprolol, xidbendol and viquidile; cytostatics such as asperline, chlorambucile, mitotane, estramustine, taxol, penclomedine and trofosfamide; hyperemic drugs such as capsaicine and methylnicotinate, lipid reducers such as nicoclonate, oxprenolol pirifibrate, simfibrate and thiadenol; spasmolytics such as aminopromazine, caronerine, difemerine, fencarbamide, tiropramide and moxaverine; testosterone derivatives such as testosterone enantate and testosterone-(4-methylpentanoate); tranquilizers such as azaperone and buramate; virustatics such as arildon; vitamin A derivates

such as retinol, retinol acetate and retinol palmitate; vitamin E derivatives such as tocopherol acetate, tocopherol succinate and tocopherol nicotinate; menadione; cholecalciferol; insecticides, pesticides and herbicides such as acephate, cyfluthrin, azinphosphomethyl, cypermethrine, substituted phenyl thiophosphates, fenclaphos, permethrine, piperonal, tetramethrine and/or trifluraline.

5. A process according to anyone of the preceding claims, characterized in that the surface characteristics of the particles are modified after homogenization in order to control the biodistribution of the particles, preferably by adsorption of polymers or by dialysis of water-soluble surfactants.

6. A process according to anyone of the preceding claims, characterized in that during cooling the dispersion is agitated, such as by means of a magnetic stirrer.

7. A process according to anyone of the preceding claims, characterized in that the dispersion medium is a pharmacologically acceptable liquid not dissolving the agent or mixture of agents, especially water, ethanol, propylene glycol, dimethyl sulfoxide (DMSO) or methyl-isobutyl-ketone, or a mixture thereof, preferably water.

8. A process according to anyone of the preceding claims, characterized in that the stabilizer or stabilizers are amphiphatic compounds, especially ionic and non-ionic surfactants, preferably naturally occurring as well as synthetic phospholipids, their hydrogenated derivatives and mixtures thereof, sphingolipids and glycosphingolipids; physiological bile salts such as sodium cholate, sodium dehydrocholate, sodium deoxycholate, sodium glycocholate and sodium taurocholate; saturated and unsaturated fatty acids or fatty alcohols; ethoxylated fatty acids or fatty alcohols and their esters and ethers; alkylaryl-polyether alcohols such as tyloxapol; esters and ethers of sugars or sugar alcohols with fatty acids or fatty alcohols; acetylated or ethoxylated mono- and diglycerides; synthetic biodegradable polymers like block co-polymers of polyoxyethylene

and polyoxypropyleneoxide; ethoxylated sorbitanesters or sorbitan-ethers; amino acids, polypeptides and proteins such as gelatine and albumin; or a combination of two or more of the above mentioned stabilizers.

- 5 9. A process according to anyone of the preceding claims,
c h a r a c t e r i z e d in that the stabilizer or stabilizers is/are a
combination of phospholipids and bile salts.
10. A process according to claim 9, c h a r a c t e r i z e d in that the
molar ratio of phospholipids to bile salts is 2:1 or above.
- 10 11. A process according to anyone of claims 1-8, c h a r a c t e r i z e d
in that the stabilizer or stabilizers is/are a combination of phospho-
lipids and sodium glycocholate in a molar ratio between 2:1 and 4:1.
12. A process according to anyone of claims 9-11,
c h a r a c t e r i z e d in that the dispersion medium contains iso-
15 tonicity agents, preferably glycerol, and/or cryoprotectants, preferably
sugars or sugar alcohols.
13. A process according to anyone of the preceding claims,
c h a r a c t e r i z e d in that the dispersion medium contains one
or more of the following additives: water-soluble or dispersable
20 stabilizers; isotonicity agents, preferably glycerol or xylitol; cryo-
protectants, preferably sucrose, glucose, maltose or trehalose; elec-
trolytes; buffers; antiflocculants, preferably sodium citrate, sodium
pyrophosphate or sodium dodecylsulfate; and preservatives.
14. A process according to anyone of the preceding claims,
25 c h a r a c t e r i z e d in that the dispersion is sterilized, preferably
by autoclaving or sterile filtration prior to cooling down the dispersion
below the recrystallization temperature of the molten lipids.
15. A process according to anyone of the preceding claims,
c h a r a c t e r i z e d in that in a subsequent step the dispersion
30 medium is reduced in volume, preferably by evaporation, or removed
by filtration, ultrafiltration or freeze-drying, thus yielding liquid-free
particles which can be reconstituted prior to use.

16. A suspension of colloidal solid lipid particles (SLPs) manufactured according to anyone of the claims 1-3 and 5-15, c h a r a c t e r i z e d in that the SLPs are lipids having melting points between approximately 30°C and 120°C, preferably constituted of mono-, di- and triglycerides of long chain fatty acids; hydrogenated vegetable oils; fatty acids and their esters; fatty alcohols and their esters and ethers; natural or synthetic waxes such as beeswax and carnauba wax; wax alcohols and their esters, sterols such as cholesterol and its esters, hard paraffins or mixtures of the above mentioned lipids.
17. A suspension of colloidal solid lipid particles (SLPs) according to claim 16, c h a r a c t e r i z e d in that the particles are stabilized by a combination of phospholipids and bile salts.
18. A suspension of colloidal solid lipid particles (SLPs) according to claim 17, c h a r a c t e r i z e d in that the molar ratio of phospholipids to bile salts is 2:1 or above.
19. A suspension of colloidal solid lipid particles (SLPs) according to claim 16, c h a r a c t e r i z e d in that the particles are stabilized by a combination of phospholipids and sodium glycocholate in a molar ratio between 2:1 and 4:1.
20. A suspension of colloidal solid lipid particles (SLPs) according to anyone of claims 17-19, c h a r a c t e r i z e d in that the dispersion medium contains isotonicity agents, preferably glycerol, and/or cryoprotectants, preferably sugars or sugar alcohols.
21. A suspension of colloidal solid lipid particles (SLPs) according to anyone of claims 16-20, c h a r a c t e r i z e d in that the SLPs are of a non- α -like crystalline modification at a temperature below the melting temperature.
22. A suspension of colloidal solid lipid particles (SLPs) according to anyone of claims 16-21, c h a r a c t e r i z e d in that the SLPs are of a non-spherical shape at a temperature below the melting temperature.

23. A suspension of colloidal solid lipid particles (SLPs) according to anyone of claims 16-22, c h a r a c t e r i z e d in that the particles are of micron or submicron size, predominantly in the size range from 20 to 500 nm.
- 5 24. A suspension of colloidal solid lipid particles (SLPs) according to anyone of claims 16-23, c h a r a c t e r i z e d in that into the SLPs are entrapped drugs or bioactive compounds which are preferably poorly water-soluble, show a low bioavailability, are badly absorbed from the intestinum and/or will be rapidly degraded in
- 10 biological environment by chemical or enzymatical processes.
25. A suspension of colloidal solid lipid particles (SLPs) according to claim 24, c h a r a c t e r i z e d in that the entrapped drugs are preferably antibiotics such as fosfomycin, fosmidomycin and rifapen-
- 15 tin; antihypertensives such as minoxidil, dihydroergotoxine and endralazine; antihypotensives such as dihydroergotamine; systemic antimycotics such as ketoconazole and griseofulvin; antiphlogistics such as indomethacin, diclofenac, ibuprofen, ketoprofen and pirpro-
- 20 fen; antiviral agents such as aciclovir, vidarabin and immunoglobulines; ACE inhibitors such as captopril and enalapril; betablockers such as propranolol, atenolol, metoprolol, pindolol, oxprenolol and labetalol; bronchodilators such as ipratropiumbromide and sobrerol;
- 25 calcium antagonists such as diltiazem, flunarizin, verapamil, nifedipin, nimodipin and nitrendipin; cardiac glycosides such as digitoxin, digoxin, methylidigoxin and acetyldigoxin; cephalosporins such as ceftizoxim, cefalexin, cefalotin and cefotaxim; cytostatics such as chlormethin, cyclophosphamid, chlorambucil, cytarabin, vincristin, mitomycin C, doxorubicin, bleomycin, cisplatin, taxol, penclomedine and estramustine; hypnotics such as flurazepam, nitrazepam and lorazepam;
- 30 psychotropic drugs such as oxazepam, diazepam and bromazepam; steroid hormones such as cortisone, hydrocortisone, prednisone, prednisolone, dexamethasone, progesterone, pregnanolone, testosterone and testosteroneundecanoat; vasodilators such as molsidomin, hydralazin and dihydralazin; cerebral vasodilators such

as dihydroergotoxin, ciclonicat and vincamin; lipophilic vitamins such as Vitamins A, D, E, K and their derivatives.

26. A suspension of particles of bioactive agents (PBAs) manufactured according to anyone of the claims 1, 2 and 4-15,

5 c h a r a c t e r i z e d in that the PBAs are drugs showing a low bioavailability and/or being badly absorbed from the intestine having melting points preferably below 100°C or the melting points of which can be decreased to below 100°C by addition of adjuvants, especially anesthetics and narcotics such as butanilicaine, fomo-
10 caine, isobutambene, lidocaine, risocaine, prilocaine, pseudococaine, tetracaine, trimecaine, tropacocaine and etomidate; anticholinergics such as metixen and profenamine; antidepressives, psychostimulants and neuroleptics such as alimenazine, binedaline, perazine, chlorpromazine, fempentadiol, fenanisol, fluanisol, mebenazine, methyl-
15 phenidate, thioridazine, toloxaton and trimipramine; antiepileptics such as dimethadion and nicethamide; antimycotics such as butoconazole, chlorphenesin, etisazole, exalamid, pecilocine and miconazole; antiphlogistics such as butibufen and ibuprofen; bronchodilators such as bamifylline; cardiovascular drugs such as alprenolol,
20 butobendine, cloridazole, hexobendine, nicofibrate, penbutolol, pirmenol, prenylamine, procaine amide, propatylnitrate, suloctidil, toliprolol, xibendol and viquidile; cytostatics such as asperline, chlorambucil, chlornaphhazine, mitotane, estramustine, taxol, penclo-
25 medine and trofosfamide; hyperemic drugs such as capsaicine and methylnicotinate; lipid reducers such as nicoclonate, oxprenolol, pirifibrate, simfibrate and thiadenol; spasmolytics such as aminopromazine, caronerine, difemerine, fencarbamide, tiropramide and moxaverine; testosterone derivatives such as testosterone enantate and testosterone-(4-methylpentanoate); tranquilizers such as azape-
30 rone and buramate; virustatics such as arildon; vitamin A derivatives such as retinol, retinol acetate and retinol palmitate; vitamin E derivatives such as tocopherol acetate, tocopherol succinate and tocopherol nicotinate; menadione; cholecalciferol; insecticides, pesticides

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and herbicides such as acephate, cyfluthrin, azinphosphomethyl, cypermethrine, substituted phenyl thiophosphates, fenclophos, permethrine, piperonal, tetramethrine and trifluraline.

- 5 27. A suspension of particles of bioactive agents (PBAs) according to claim 26 or manufactured according to anyone of the claims 4-15, characterized in that the PBAs are water-insoluble at the temperature of emulsion preparation and will not be solubilized by the excess of stabilizers or form micelles by themselves the particle size of PBAs remaining unchanged before and after cooling to room temperature.
- 10 28. A suspension of particles of bioactive agents (PBAs) according to claim 26 or manufactured according to anyone of claims 4-15, characterized in that the PBAs are partly water-soluble at the temperature of emulsion preparation and/or are able to form mixed micelles by the excess of stabilizers and/or form micelles by themselves leading to an increase of particle size after cooling to room temperature due to crystal growth and/or precipitation of dissolved bioactive agent and/or due to mass transport from smaller to larger particles.
- 15 29. Liquid-free particles manufactured by removing the dispersion medium from a suspension according to anyone of claims 16-25, preferably by filtration, ultrafiltration or freeze-drying.
- 20 30. Liquid-free particles manufactured by removing the dispersion medium from a suspension according to anyone of claims 26-28, preferably by filtration, ultrafiltration or freeze-drying.
- 25 31. Use of a suspension according to anyone of claims 16-28 or liquid-free particles according to claim 29 or 30, but not containing insecticides, pesticides or herbicides, for therapeutic purposes in a living human or animal body.
- 30 32. A suspension according to anyone of the claims 16-28 or liquid-free particles according to claim 29 or 30 for use as a medicament.

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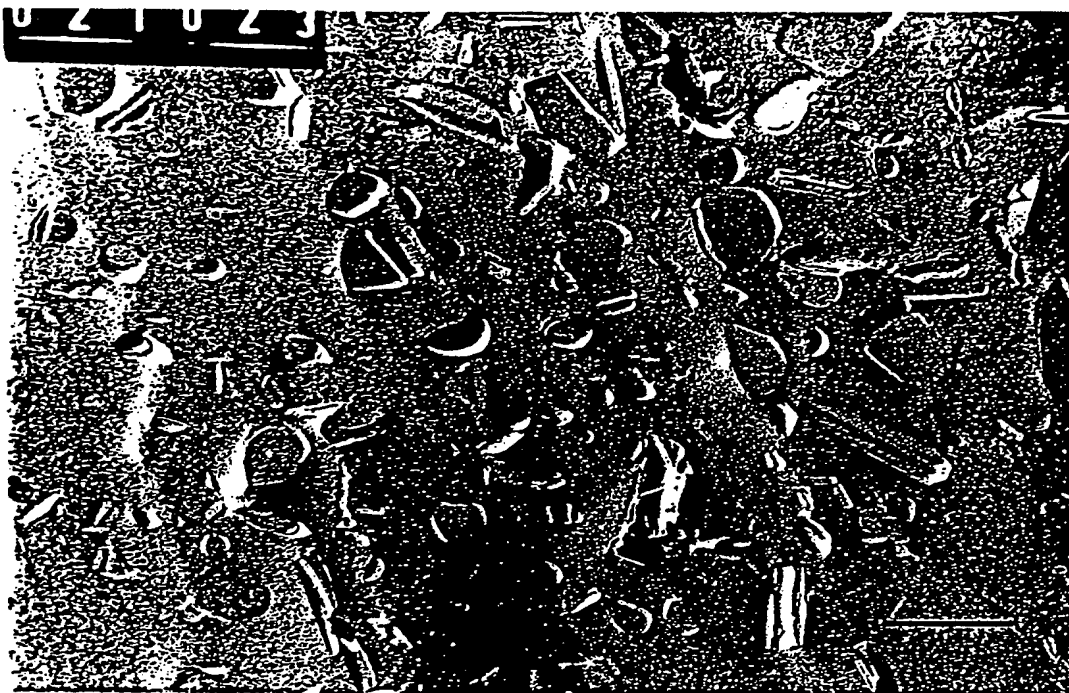


Fig. 1

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DIFFERENTIAL SCANNING CALORIMETRY

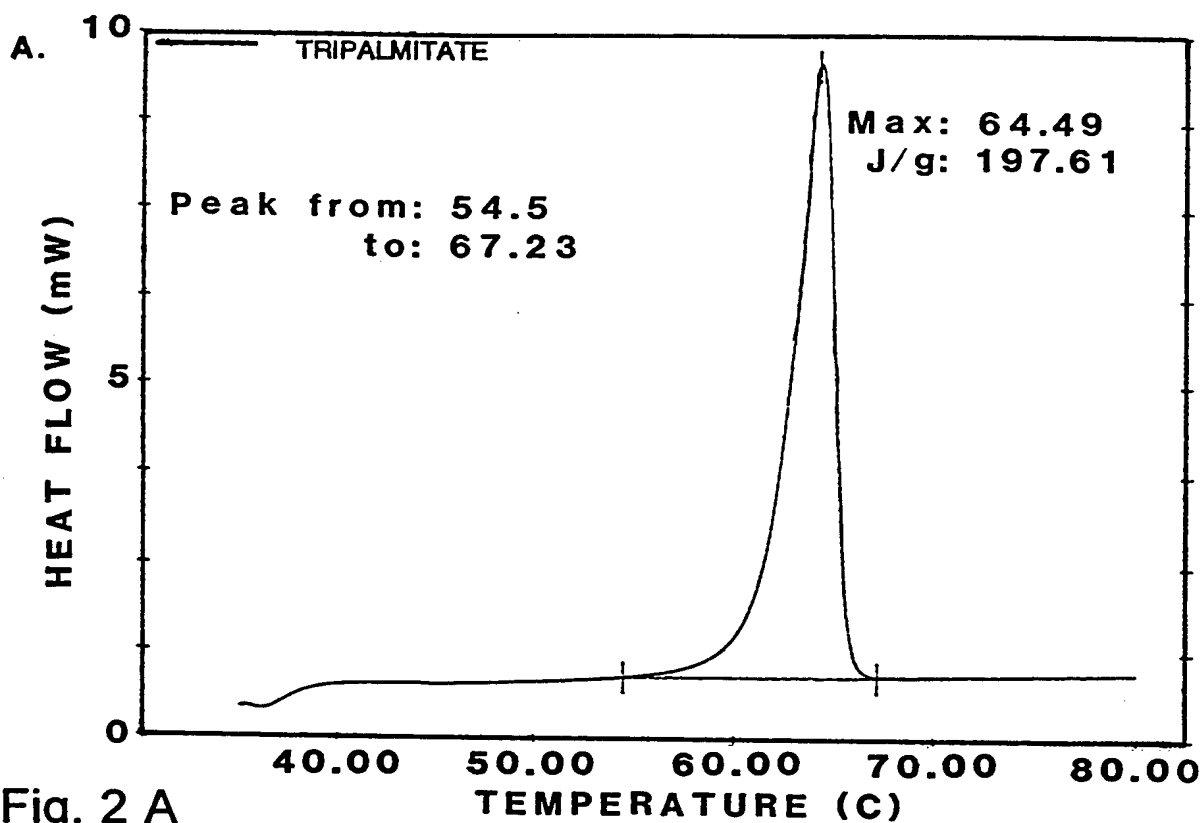


Fig. 2 A

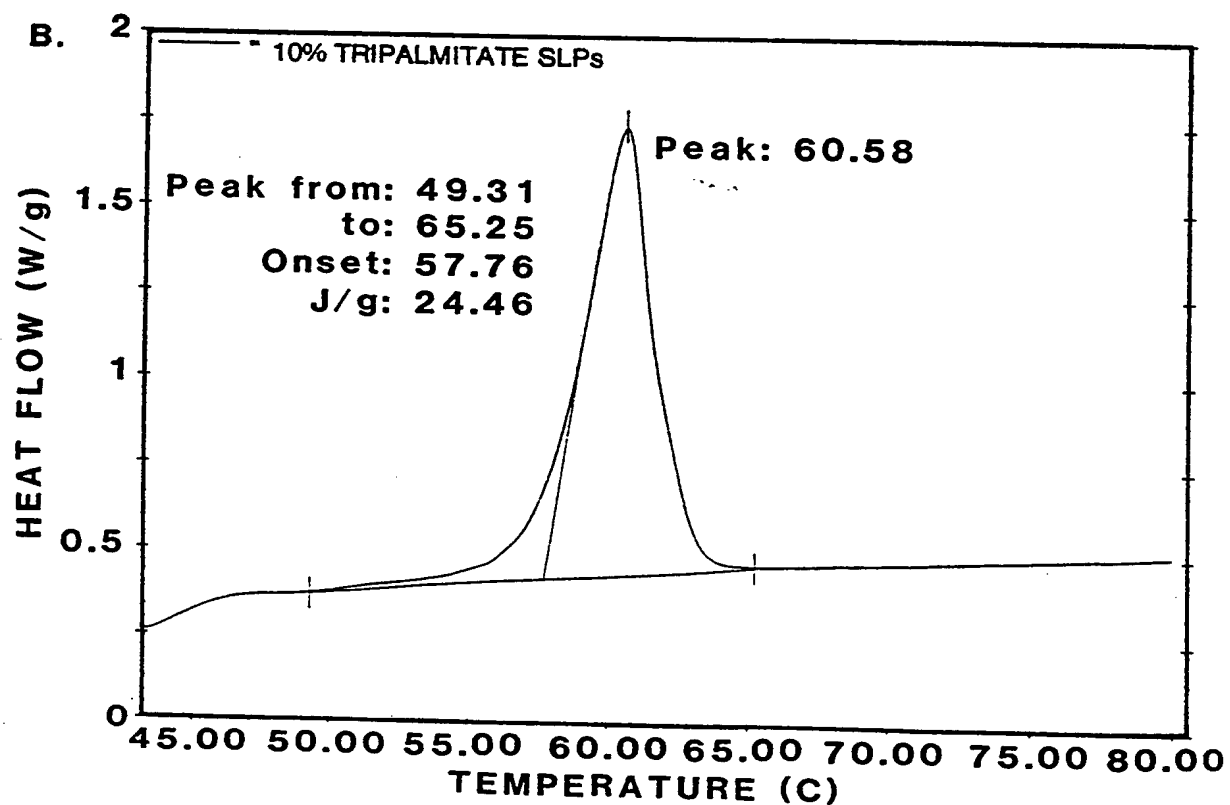


Fig. 2 B

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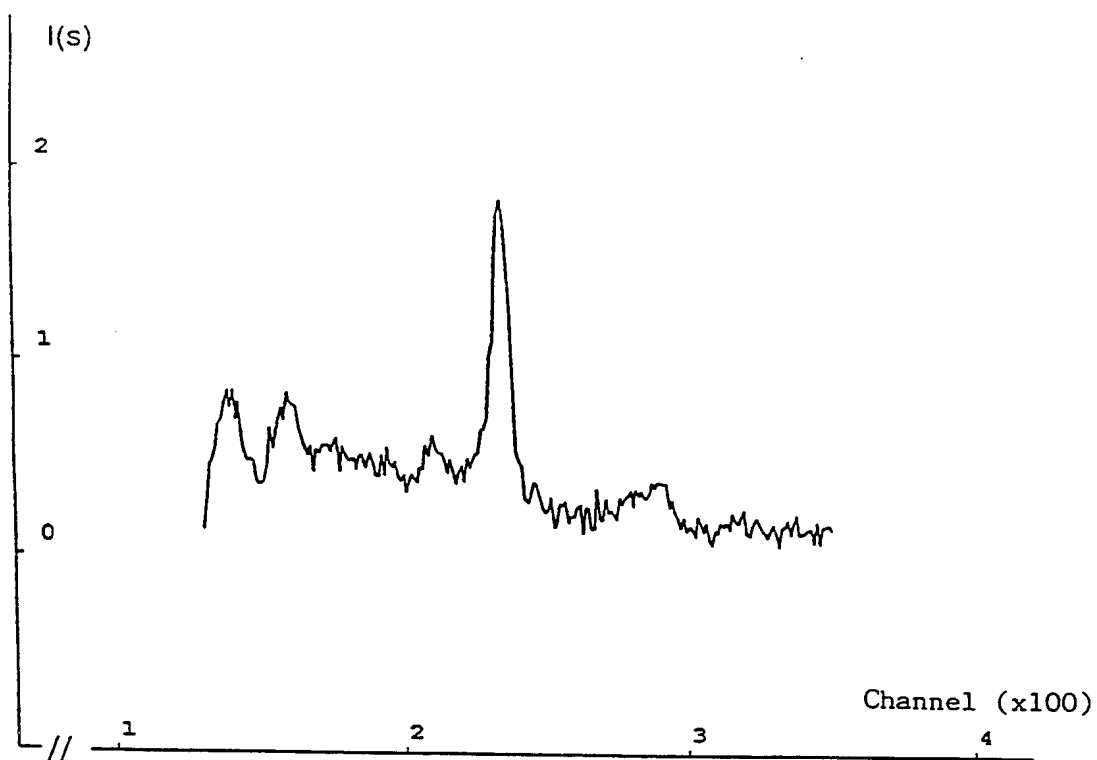


Fig. 3

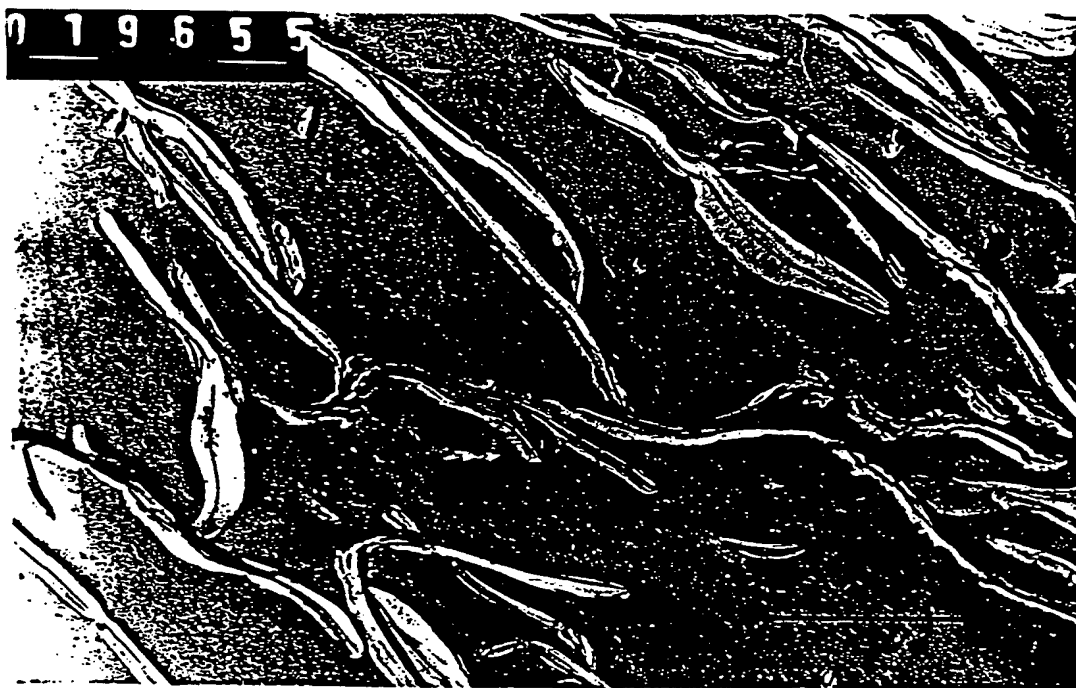


Fig. 4

Malvern Multi-Angle PCS data analysis

10% Tripalmitate SLP dispersion

Angles 50/ 70/ 90/ 110/ 130/ degrees

File ml9103ma.mad

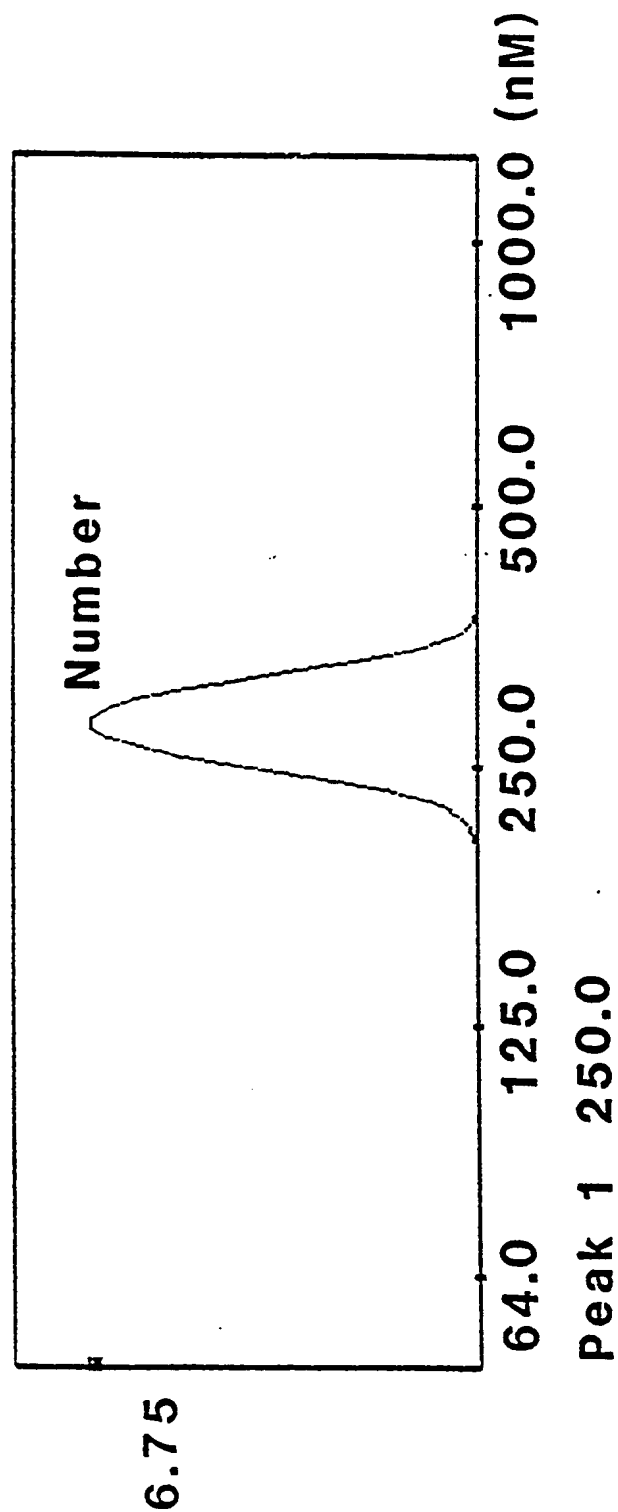


Fig. 5

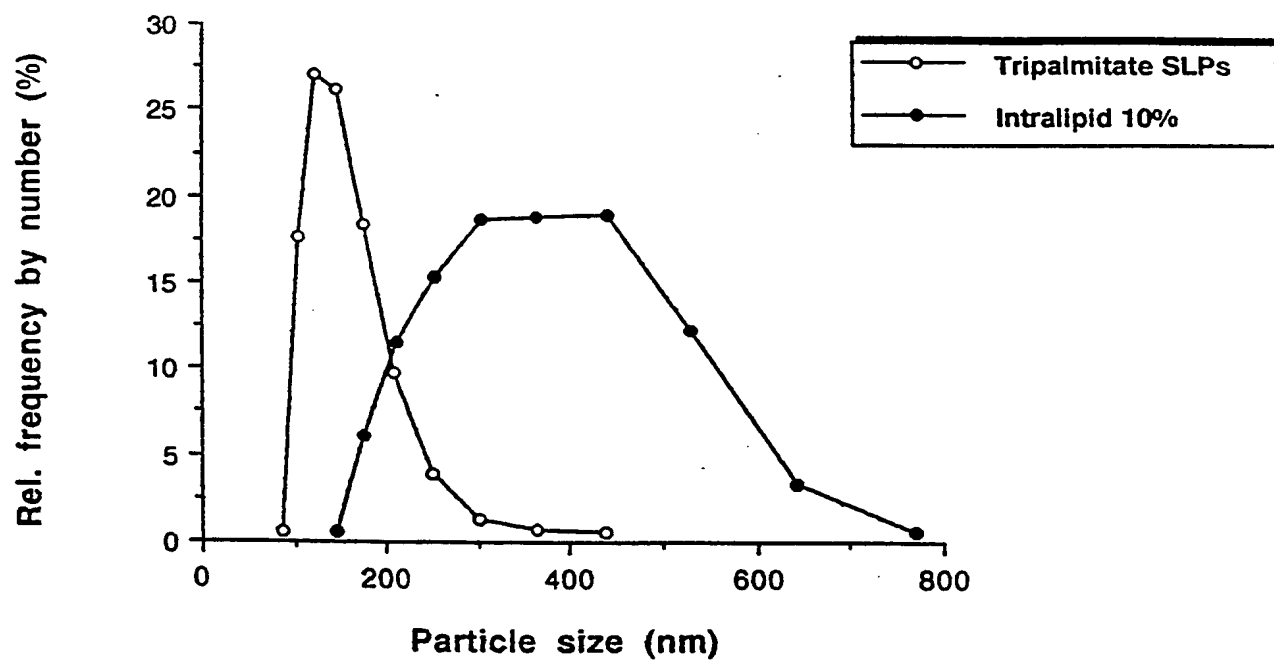


Fig. 6

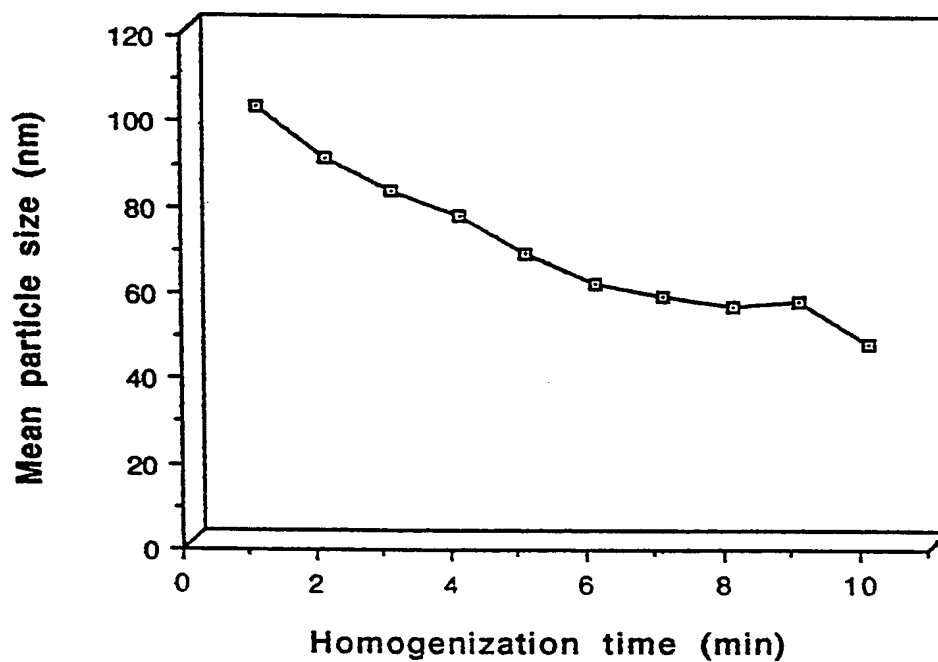


Fig. 7

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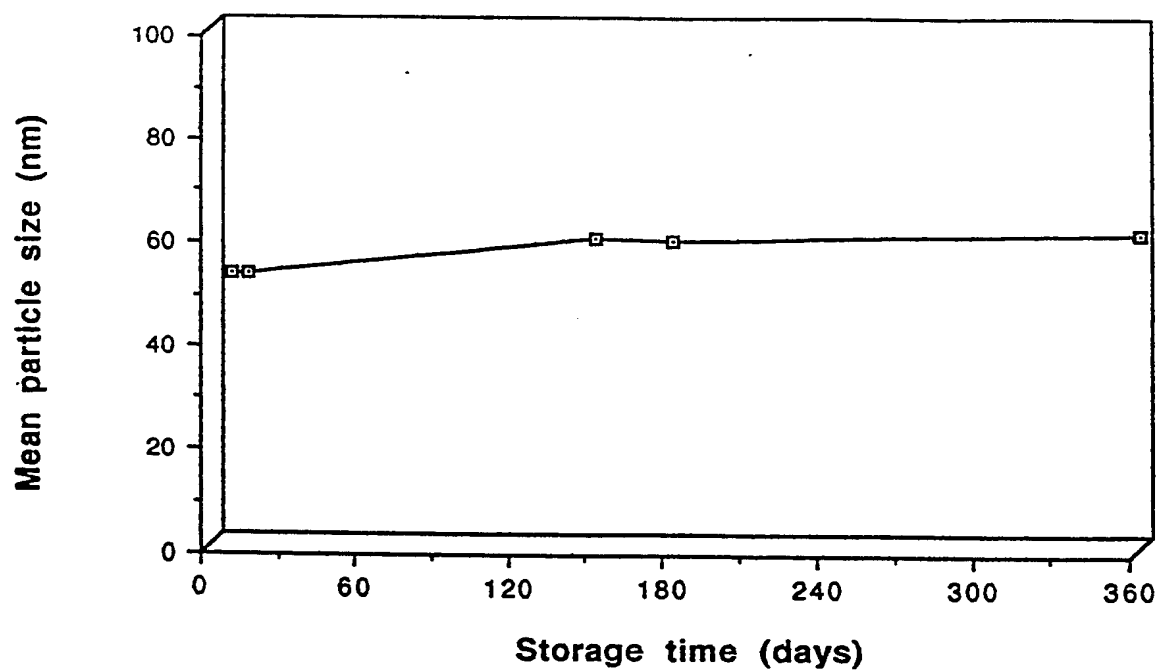


Fig. 8

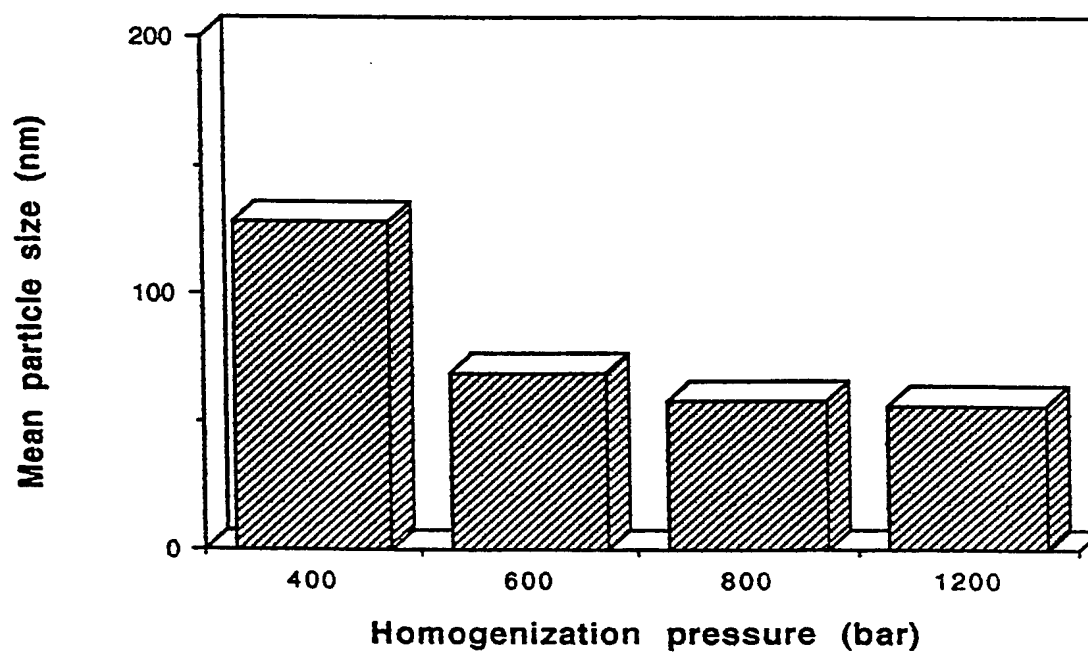


Fig. 9

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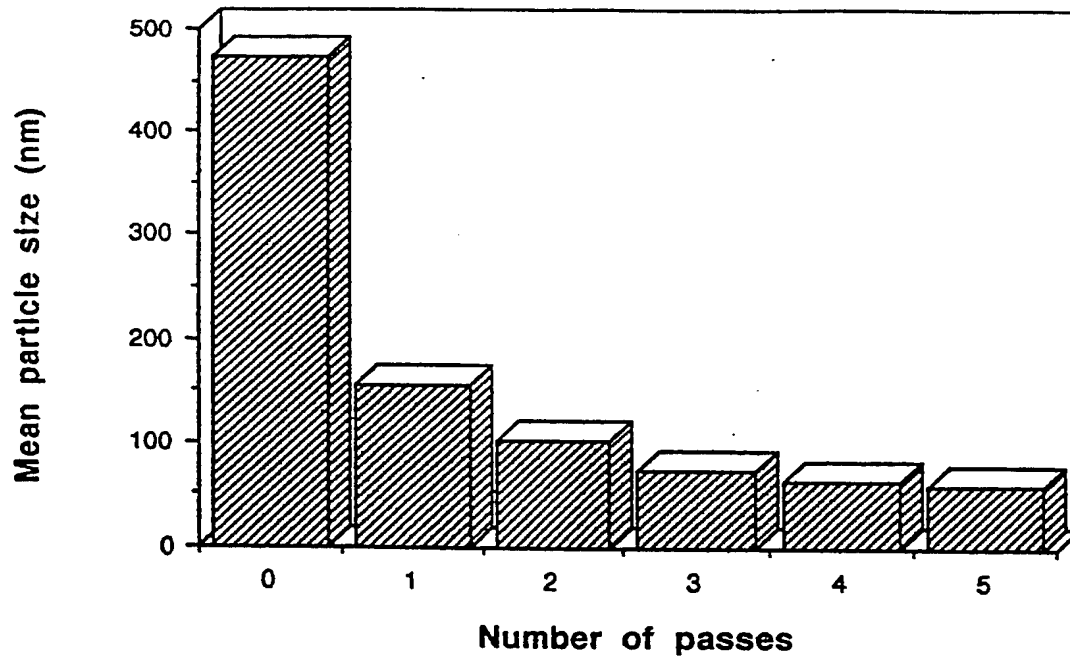


Fig. 10

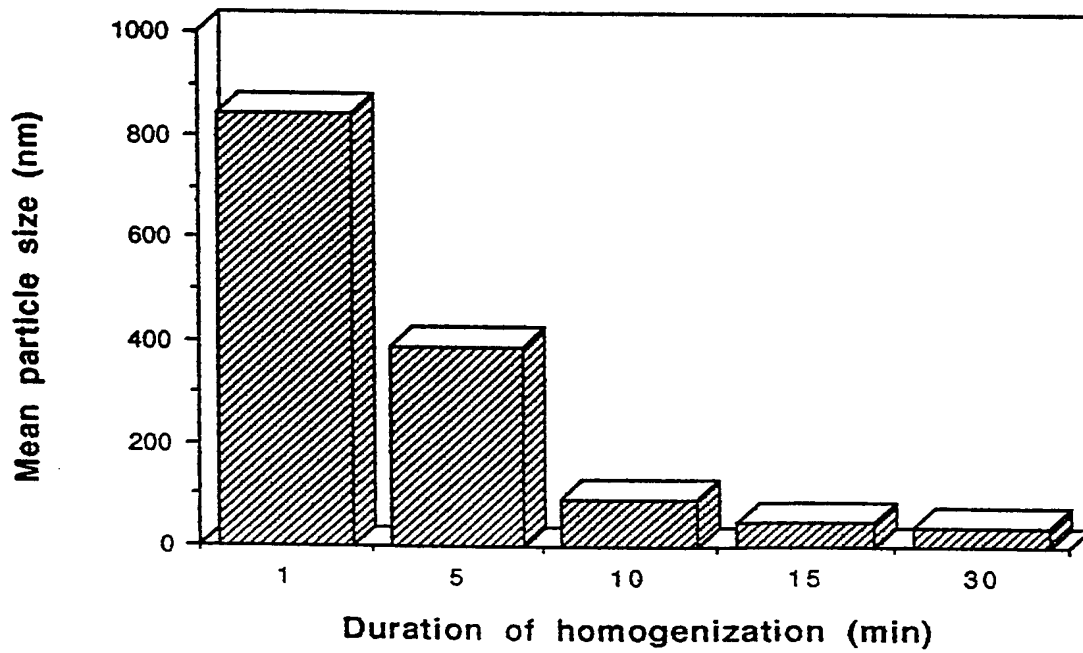


Fig. 11

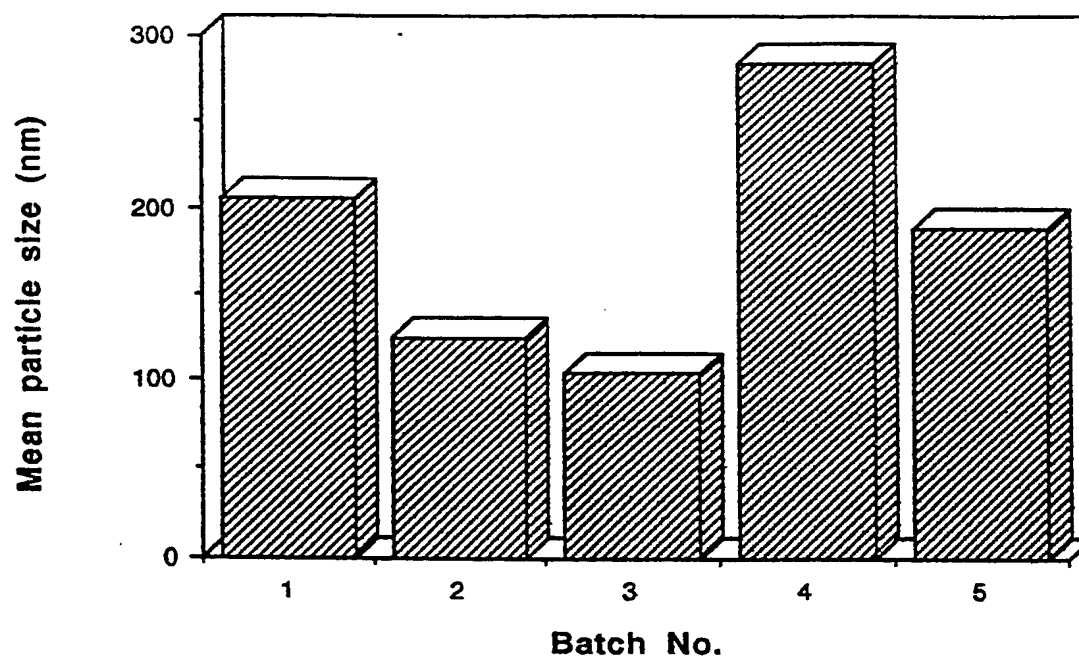


Fig. 12

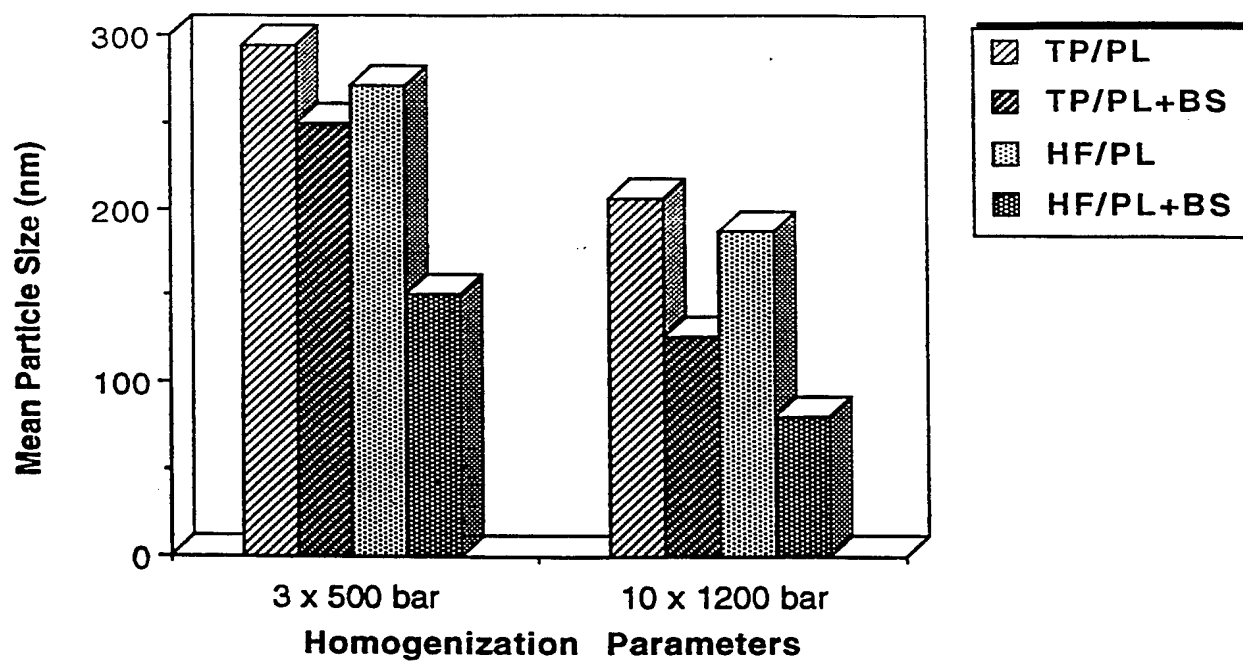


Fig. 13

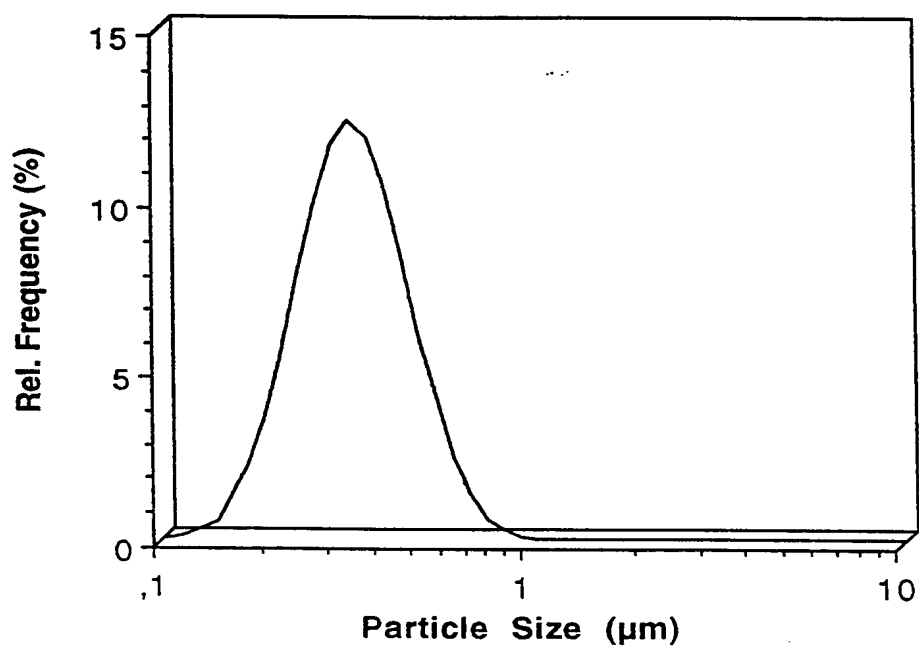


Fig. 14

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SYNCHROTRON RADIATION WIDE ANGLE X-RAY DIFFRACTION

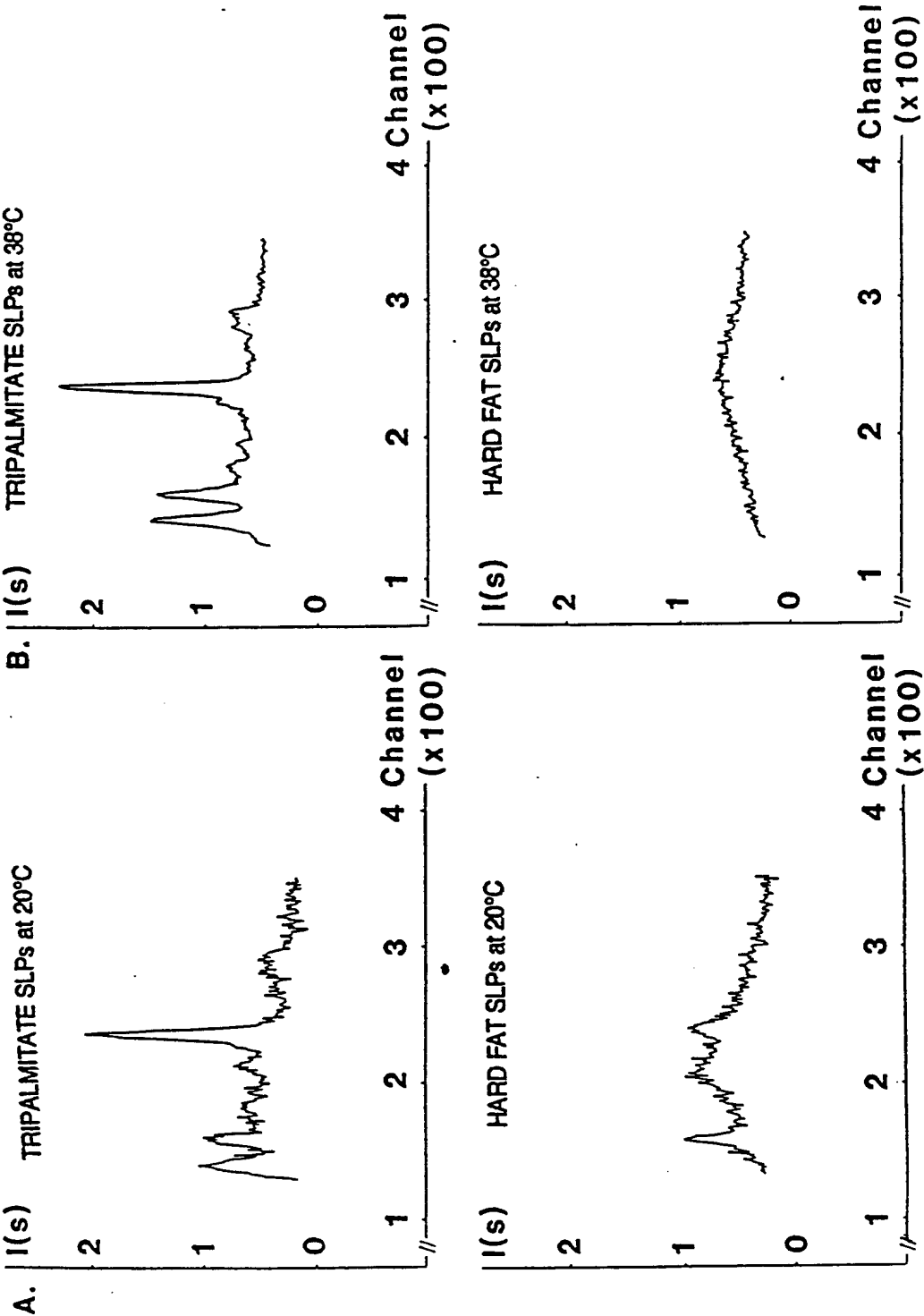


Fig. 15

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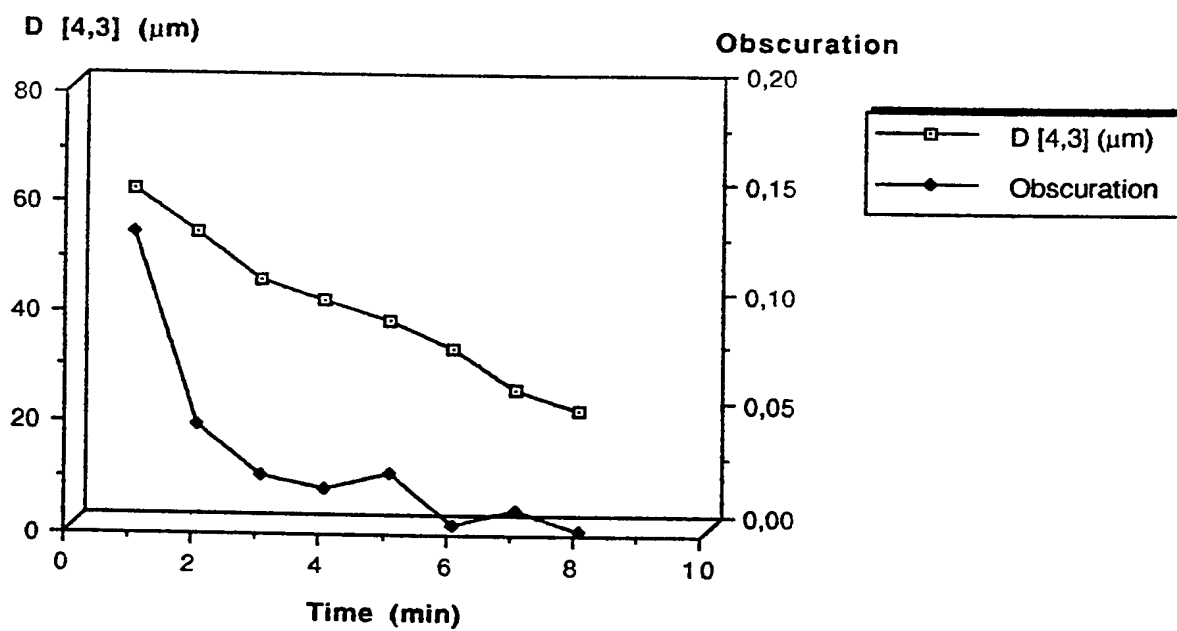


Fig. 16



Fig. 17



Fig. 18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00185

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 9/10, A61K 9/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, B01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBASE, CA SEARCH, MEDLINE, WPI, WPIL, CLAIMS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9107171 (NOVA PHARMACEUTICAL CORPORATION), 30 May 1991 (30.05.91), page 7 - page 22, claims --	1-32
X	EP, A2, 0167825 (DR. RENTSCHLER ARZNEIMITTEL GMBH & CO), 15 January 1986 (15.01.86), page 14, line 13 - page 16, line 12, claims --	1-32

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00185

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>Dialog Information Services, File 73, Embase, Dialog accession no. 8396396, Embase accession no. 92072268, Bodmeier R. et al, "Process and formulation variables in the preparation of wax microparticles by a melt dispersion technique. I. Oil-in-water technique for water-insoluble drugs". J. Microencapsulation (United Kingdom), 1992, 9/1 (89-98)</p> <p style="text-align: center;">--</p>	1-32
A	<p>US, A, 5118511 (DIETER HORN ET AL), 2 June 1992 (02.06.92)</p> <p style="text-align: center;">-- -----</p>	1-32

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/05/94

International application No.

PCT/SE 94/00185

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9107171	30/05/91	AU-A- 6950091 CA-A- 2068216 EP-A- 0502119	13/06/91 14/05/91 09/09/92
EP-A2- 0167825	15/01/86	SE-T3- 0167825 DE-A- 3421468 JP-A- 61056122 US-A- 4880634	19/12/85 20/03/86 14/11/89
US-A- 5118511	02/06/92	DE-A- 3702029 DE-A- 3869629 EP-A,B- 0276735 SE-T3- 0276735	04/08/88 07/05/92 03/08/88

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(54) Title: ANTI-ANGIOGENIC COMPOSITIONS AND METHODS OF USE		
(57) Abstract The present invention provides compositions comprising an anti-angiogenic factor, and a polymeric carrier. Representative examples of anti-angiogenic factors include Anti-Invasive Factor, Retinoic acids and derivatives thereof, and taxol. Also provided are methods for embolizing blood vessels, and eliminating biliary, urethral, esophageal, and tracheal/bronchial obstructions.		

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Description

ANTI-ANGIOGENIC COMPOSITIONS AND METHODS OF USE

5 Technical Field

The present invention relates generally to compositions and methods for treating cancer and other angiogenic-dependent diseases, and more specifically, to compositions comprising anti-angiogenic factors and polymeric carriers, stents which have been coated with such compositions, as well as
10 methods for utilizing these stents and compositions.

Background Of The Invention

Cancer is the second leading cause of death in the United States, and accounts for over one fifth of the total mortality. Briefly, cancer is
15 characterized by the uncontrolled division of a population of cells which, most typically, leads to the formation of one or more tumors. Although cancer is generally more readily diagnosed than in the past, many forms, even if detected early, are still incurable.

A variety of methods are presently utilized to treat cancer,
20 including for example various surgical procedures. If treated with surgery alone, however, many patients (particularly those with certain types of cancer, such as breast, brain, colon and hepatic cancer) will experience recurrence of the cancer. In addition to surgery, many cancers are also treated with a combination of therapies involving cytotoxic chemotherapeutic drugs (e.g.,
25 vincristine, vinblastine, cisplatin, methotrexate, 5-FU, etc.) and/or radiation therapy. One difficulty with this approach, however, is that radiotherapeutic and chemotherapeutic agents are toxic to normal tissues, and often create life-threatening side effects. In addition, these approaches often have extremely high failure/remission rates.

30 In addition to surgical, chemo and radiation therapies, others have attempted to utilize an individual's own immune system in order to eliminate cancerous cells. For example, some have suggested the use of bacterial or viral components as adjuvants in order to stimulate the immune system to destroy tumor cells. (See generally "Principles of Cancer Biotherapy," Oldham (ed.),
35 Raven Press, New York, 1987.) Such agents have generally been useful as adjuvants and as nonspecific stimulants in animal tumor models, but have not as of yet proved to be generally effective in humans.

Lymphokines have also been utilized in the treatment of cancer. Briefly, lymphokines are secreted by a variety of cells, and generally have an effect on specific cells in the generation of an immune response. Examples of lymphokines include Interleukins (IL)-1, -2, -3, and -4, as well as colony stimulating factors such as G-CSF, GM-CSF, and M-CSF. Recently, one group has
5 utilized IL-2 to stimulate peripheral blood cells in order to expand and produce large quantities of cells which are cytotoxic to tumor cells (Rosenberg et al., *N. Engl. J. Med.* 313:1485-1492, 1985).

Others have suggested the use of antibodies in the treatment of
10 cancer. Briefly, antibodies may be developed which recognize certain cell surface antigens that are either unique, or more prevalent on cancer cells compared to normal cells. These antibodies, or "magic bullets," may be utilized either alone or conjugated with a toxin in order to specifically target and kill tumor cells (Dillman, "Antibody Therapy," *Principles of Cancer Biotherapy*,
15 Oldham (ed.), Raven Press, Ltd., New York, 1987). However, one difficulty is that most monoclonal antibodies are of murine origin, and thus hypersensitivity against the murine antibody may limit its efficacy, particularly after repeated therapies. Common side effects include fever, sweats and chills, skin rashes, arthritis, and nerve palsies.

20 One additional difficulty of present methods is that local recurrence and local disease control remains a major challenge in the treatment of malignancy. In particular, a total of 630,000 patients annually (in the U.S.) have localized disease (no evidence of distant metastatic spread) at the time of presentation; this represents 64% of all those patients diagnosed with
25 malignancy (this does not include nonmelanoma skin cancer or carcinoma *in situ*). For the vast majority of these patients, surgical resection of the disease represents the greatest chance for a cure and indeed 428,000 will be cured after the initial treatment - 428,000. Unfortunately, 202,000 (or 32% of all patients with localized disease) will relapse after the initial treatment. Of those who
30 relapse, the number who will relapse due to local recurrence of the disease amounts to 133,000 patients annually (or 21% of all those with localized disease). The number who will relapse due to distant metastases of the disease is 68,000 patients annually (11% of all those with localized disease). Another 102,139 patients annually will die as a direct result of an inability to control the
35 local growth of the disease.

Nowhere is this problem more evident than in breast cancer, which affects 186,000 women annually in the U.S. and whose mortality rate has

remained unchanged for 50 years. Surgical resection of the disease through radical mastectomy, modified radical mastectomy, or lumpectomy remains the mainstay of treatment for this condition. Unfortunately, 39% of those treated with lumpectomy alone will develop a local recurrence of the disease, and
5 surprisingly, so will 25% of those in which the resection margin is found to be clear of tumor histologically. As many as 90% of these local recurrences will occur within 2 cm of the previous excision site.

Similarly, in 1991, over 113,000 deaths and 238,600 new cases of liver metastasis were reported in North America alone. The mean survival time
10 for patients with liver metastases is only 6.6 months once liver lesions have developed. Non-surgical treatment for hepatic metastases include systemic chemotherapy, radiation, chemoembolization, hepatic arterial chemotherapy, and intraarterial radiation. However, despite evidence that such treatments can transiently decrease the size of the hepatic lesions (*e.g.*, systemic chemotherapy
15 and hepatic arterial chemotherapy initially reduces lesions in 15-20%, and 80% of patients, respectively), the lesions invariably reoccur. Surgical resection of liver metastases represents the only possibility for a cure, but such a procedure is possible in only 5% of patients with metastases, and in only 15-20% of patients with primary hepatic cancer.

20 One method that has been attempted for the treatment of tumors with limited success is therapeutic embolization. Briefly, blood vessels which nourish a tumor are deliberately blocked by injection of an embolic material into the vessels. A variety of materials have been attempted in this regard, including autologous substances such as fat, blood clot, and chopped muscle
25 fragments, as well as artificial materials such as wool, cotton, steel balls, plastic or glass beads, tantalum powder, silicone compounds, radioactive particles, sterile absorbable gelatin sponge (Sterispon, Gelfoam), oxidized cellulose (Oxycel), steel coils, alcohol, lyophilized human dura mater (Lyodura), microfibrillar collagen (Avitene), collagen fibrils (Tachotop), polyvinyl alcohol
30 sponge (PVA; Ivalon), Barium-impregnated silicon spheres (Biss) and detachable balloons. The size of liver metastases may be temporarily decreased utilizing such methods, but tumors typically respond by causing the growth of new blood vessels into the tumor.

A related problem to tumor formation is the development of
35 cancerous blockages which inhibit the flow of material through body passageways, such as the bile ducts, trachea, esophagus, vasculature and urethra. One device, the stent, has been developed in order to hold open passageways

which have been blocked by tumors or other substances. Representative examples of common stents include the Wallstent, Strecker stent, Gianturco stent and the Palmaz stent. The major problem with stents, however, is that they do not prevent the ingrowth of tumor or inflammatory material through the
5 interstices of the stent. If this material reaches the inside of a stent and compromises the stent lumen, it may result in blockage of the body passageway into which it has been inserted. In addition, presence of a stent in the body may induce reactive or inflammatory tissue (e.g., blood vessels, fibroblasts, white blood cells) to enter the stent lumen, resulting in partial or complete closure of
10 the stent.

The present invention provides compositions and methods suitable for treating cancers and other angiogenesis-dependent diseases which address the problems associated with the procedures discussed above, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides anti-angiogenic compositions, as well as methods and devices which utilize such compositions for the treatment of cancer and other angiogenesis-dependent diseases. Within
20 one aspect of the present invention, compositions are provided (hereinafter referred to as "anti-angiogenic compositions") comprising (a) an anti-angiogenic factor and (b) a polymeric carrier. A wide variety of molecules may be utilized within the scope of the present invention as anti-angiogenic factors, including for example Anti-Invasive Factor, retinoic acids and their derivatives, taxol,
25 taxol analogues and taxol derivatives, and members of the group consisting of Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1 and Plasminogen Activator Inhibitor-2. Similarly, a wide variety of polymeric carriers may be utilized, representative examples of which include poly(ethylene-vinyl acetate)
30 crosslinked with 40% vinyl acetate, poly (lactic-co-glycolic acid), polycaprolactone polylactic acid, copolymers of poly(ethylene-vinyl acetate) crosslinked with 40% vinyl acetate and polylactic acid, and copolymers of polylactic acid and polycaprolactone. Within one embodiment of the invention, the composition has an average size of 15 to 200 μm .

35 Within another aspect of the present invention methods for embolizing a blood vessel are provided, comprising the step of delivering into the vessel a therapeutically effective amount of an anti-angiogenic composition

(as described above), such that the blood vessel is effectively occluded. Within one embodiment, the anti-angiogenic composition is delivered to a blood vessel which nourishes a tumor.

5 Within yet another aspect of the present invention, stents are provided comprising a generally tubular structure, the surface being coated with one or more anti-angiogenic compositions. Within other aspects of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the passageway is expanded. Within various embodiments of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway; for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra; for eliminating esophageal obstructions, 10 comprising inserting an esophageal stent into an esophagus; and for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi. In each of these embodiments, the stent has a generally tubular structure, the surface of which is coated with an anti-angiogenic composition as described above.

20 Within another aspect of the present invention, methods are provided for treating tumor excision sites, comprising administering an anti-angiogenic composition as described above to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within yet another aspect of the invention, methods for treating corneal neovascularization are 25 provided, comprising the step of administering a therapeutically effective amount of an anti-angiogenic composition as described above to the cornea, such that the formation of blood vessels is inhibited. Within one embodiment, the anti-angiogenic composition further comprises a topical corticosteroid.

30 Within another aspect of the present invention, methods are provided for inhibiting angiogenesis in patients with non-tumorigenic, angiogenesis-dependent diseases, comprising administering a therapeutically effective amount of a composition comprising taxol to a patient with a non-tumorigenic angiogenesis-dependent disease, such that the formation of new 35 blood vessels is inhibited. Within other aspects, methods are provided for embolizing blood vessels in non-tumorigenic, angiogenesis-dependent diseases,

comprising delivering to the vessel a therapeutically effective amount of a composition comprising taxol, such that the blood vessel is effectively occluded.

Within yet other aspects of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a
5 stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with a composition comprising taxol, such that the passageway is expanded. Within various embodiments of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway; for eliminating urethral obstructions,
10 comprising inserting a urethral stent into a urethra; for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus; and for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi. Within each of these embodiments the stent has a generally tubular structure, the surface of the
15 structure being coated with a composition comprising taxol.

Within another aspect of the present invention, methods are provided for treating a tumor excision site, comprising administering a composition comprising taxol to the resection margin of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new
20 blood vessels at the site is inhibited. Within other aspects, methods are provided for treating corneal neovascularization, comprising administering a therapeutically effective amount of a composition comprising taxol to the cornea, such that the formation of new vessels is inhibited.

Within yet another aspect of the invention, pharmaceutical
25 products are provided, comprising (a) taxol, in a container, and (b) a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the taxol, for human or veterinary administration to treat non-tumorigenic angiogenesis-dependent diseases. Briefly, Federal Law requires that the use of a pharmaceutical agent in the
30 therapy of humans be approved by an agency of the Federal government. Responsibility for enforcement (in the United States) is with the Food and Drug Administration, which issues appropriate regulations for securing such approval, detailed in 21 U.S.C. §§ 301-392. Regulation for biological materials comprising
35 products made from the tissues of animals, is also provided under 42 U.S.C. § 262. Similar approval is required by most countries, although, regulations may vary from country to country.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions, and are incorporated by
5 reference in their entirety.

Brief Description of the Drawings

Figure 1A is a photograph which shows a shell-less egg culture on day 6. Figure 1B is a digitized computer-displayed image taken with a
10 stereomicroscope of living, unstained capillaries (1040x). Figure 1C is a corrosion casting which shows CAM microvasculature that are fed by larger, underlying vessels (arrows; 1300x). Figure 1D depicts a 0.5 mm thick plastic section cut transversely through the CAM, and recorded at the light microscope level. This photograph shows the composition of the CAM, including an outer
15 double-layered ectoderm (Ec), a mesoderm (M) containing capillaries (arrows) and scattered adventitial cells, and a single layered endoderm (En) (400x). Figure 1E is a photograph at the electron microscope level (3500x) wherein typical capillary structure is presented showing thin-walled endothelial cells. (arrowheads) and an associated pericyte.

20 Figures 2A, 2B, 2C and 2D are a series of digitized images of four different, unstained CAMs taken after a 48 hour exposure to taxol.

Figures 3A, 3B and 3C are a series of photographs of 0.5 mm thick plastic sections transversely cut through a taxol-treated CAM at three different locations within the avascular zone.

25 Figures 4A, 4B and 4C are series of electron micrographs which were taken from locations similar to that of Figures 3A, 3B and 3C (respectively) above.

Figure 5 is a bar graph which depicts the size distribution of microspheres by number (5% ELVAX with 10 mg sodium suramin into 5%
30 PVA).

Figure 6 is a bar graph which depicts the size distribution of microspheres by weight (5% ELVAX with 10 mg sodium suramin into 5% PVA).

35 Figure 7 is a line graph which depicts the weight of encapsulation of Sodium Suramin in 1 ml of 5% ELVAX.

Figure 8 is a line graph which depicts the percent of encapsulation of Sodium Suramin in ELVAX.

Figure 9 is a bar graph which depicts the size distribution of 5% ELVAX microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

5 Figure 10 is a bar graph which depicts the size distribution by weight of 5% PLL microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

Figure 11 is a bar graph which depicts the size distribution by number of 5% PLL microspheres containing 10 mg sodium suramin made in 5% PVA containing 10% NaCl.

10 Figure 12 is a line graph which depicts the time course of sodium suramin release.

Figure 13 is an illustration of a representative embodiment of hepatic tumor embolization.

15 Figure 14 is an illustration of the insertion of a representative stent coated with an anti-angiogenic composition of the present invention.

Figure 15A is a graph which shows the effect of the EVA:PLA polymer blend ratio upon aggregation of microspheres. Figure 15B is a scanning electron micrograph which shows the size of "small" microspheres. Figure 15C is a scanning electron micrograph which shows the size of "large" 20 microspheres. Figure 15D is a graph which depicts the time course of *in vitro* taxol release from 0.6% w/v taxol-loaded 50:50 EVA:PLA polymer blend microspheres into phosphate buffered saline (pH 7.4) at 37°C. Open circles are "small" sized microspheres, and closed circles are "large" sized microspheres. Figure 15E is a photograph of a CAM which shows the results of taxol release 25 by microspheres ("MS"). Figure 15F is a photograph similar to that of 15E at increased magnification.

Figure 16 is a graph which shows release rate profiles from polycaprolactone microspheres containing 1%, 2%, 5% or 10% taxol into phosphate buffered saline at 37°C. Figure 16B is a photograph which shows a 30 CAM treated with control microspheres. Figure 16C is a photograph which shows a CAM treated with 5% taxol loaded microspheres.

Figures 17A and 17B, respectively, are two graphs which show the release of taxol from EVA films, and the percent taxol remaining in those same films over time. Figure 17C is a graph which shows the swelling of EVA/F127 35 films with no taxol over time. Figure 17D is a graph which shows the swelling of EVA/Span 80 films with no taxol over time. Figure 17E is a graph which depicts a stress vs. strain curve for various EVA/F127 blends.

Figures 18A and 18B are two graphs which show the melting point of PCL/MePEG polymer blends as a function of % MePEG in the formulation (18A), and the percent increase in time needed for PCL paste at 60°C to begin to solidify as a function of the amount of MePEG in the formulation (18B).
5 Figure 18C is a graph which depicts the brittleness of varying PCL/MePEG polymer blends. Figure 18D is a graph which shows the percent weight change over time for polymer blends of various MePEG concentrations. Figure 18E is a graph which depicts the rate of taxol release over time from various polymer blends loaded with 1% taxol. Figures 18F and 18G are graphs which depict the
10 effect of varying quantities of taxol on the total amount of taxol released from a 20%MePEG/PCL blend. Figure 18H is a graph which depicts the effect of MePEG on the tensile strength of a MePEG/PCL polymer.

Figure 19A is a photograph which shows control (unloaded) thermopaste on a CAM. Figure 19B is a photograph of 20% taxol-loaded
15 thermopaste on a CAM.

Figures 20A and 20B are two photographs of a CAM having a tumor treated with control (unloaded) thermopaste. Figures 20C and 20D are two photographs of a CAM having a tumor treated with taxol-loaded thermopaste.

20 Figure 21A is a graph which shows the effect of taxol/PCL on tumor growth. Figures 21B and 21C are two photographs which show the effect of control, 10%, and 20% taxol-loaded thermopaste on tumor growth.

Figure 22A is a photograph of synovium from a PBS injected joint. Figure 22B is a photograph of synovium from a microsphere injected joint.
25 Figure 22C is a photograph of cartilage from joints injected with PBS, and Figure 22D is a photograph of cartilage from joints injected with microspheres.

Detailed Description of the Invention

30 As noted above, the present invention provides methods and compositions which utilize anti-angiogenic factors. Briefly, within the context of the present invention, anti-angiogenic factors should be understood to include any protein, peptide, chemical or other molecule which acts to inhibit vascular growth. A variety of methods may be readily utilized to determine the anti-
35 angiogenic activity of a given factor, including for example, chick chorioallantoic membrane ("CAM") assays. Briefly, as described in more detail below in Examples 2A and 2C, a portion of the shell from a freshly fertilized chicken egg

is removed, and a methyl cellulose disk containing a sample of the anti-angiogenic factor to be tested is placed on the membrane. After several days (e.g., 48 hours), inhibition of vascular growth by the sample to be tested may be readily determined by visualization of the chick chorioallantoic membrane in the region surrounding the methyl cellulose disk. Inhibition of vascular growth may also be determined quantitatively, for example, by determining the number and size of blood vessels surrounding the methyl cellulose disk, as compared to a control methyl cellulose disk. Particularly preferred anti-angiogenic factors suitable for use within the present invention completely inhibit the formation of new blood vessels in the assay described above.

A variety of assays may also be utilized to determine the efficacy of anti-angiogenic factors *in vivo*, including for example, mouse models which have been developed for this purpose (see Roberston et al., *Cancer. Res.* 51:1339-1344, 1991). In addition, a variety of representative *in vivo* assays relating to various aspects of the inventions described herein have been described in more detail below in Examples 5 to 7, and 17 to 19.

As noted above, the present invention provides compositions comprising an anti-angiogenic factor and a polymeric carrier. Briefly, a wide variety of anti-angiogenic factors may be readily utilized within the context of the present invention. Representative examples include Anti-Invasive Factor, retinoic acid and derivatives thereof, taxol, and members of the group consisting of Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1 and Plasminogen Activator Inhibitor-2. These and other anti-angiogenic factors will be discussed in more detail below.

Briefly, Anti-Invasive Factor, or "AIF" which is prepared from extracts of cartilage, is known to contain constituents which are responsible for inhibiting the growth of new blood vessels. These constituents comprise a family of 7 low molecular weight proteins (<50,000 daltons) (Kuettnner and Pauli, "Inhibition of neovascularization by a cartilage factor" in *Development of the Vascular System*, Pitman Books (Ciba Foundation Symposium 100), pp. 163-173, 1983), including a variety of proteins which have inhibitory effects against a variety of proteases (Eisentein et al, *Am. J. Pathol.* 81:337-346, 1975; Langer et al., *Science* 193:70-72, 1976; and Horton et al., *Science* 199:1342-1345, 1978). AIF suitable for use within the present invention may be readily prepared utilizing techniques known in the art (e.g., Eisentein et al, *supra*; Kuettnner and Pauli, *supra*; and Langer et al., *supra*). Purified constituents of AIF such as

Cartilage-Derived Inhibitor ("CDI") (see Moses et al., *Science* 248:1408-1410, 1990) may also be readily prepared and utilized within the context of the present invention.

5 Retinoic acids alter the metabolism of extracellular matrix components, resulting in the inhibition of angiogenesis. Addition of proline analogs, angiostatic steroids, or heparin may be utilized in order to synergistically increase the anti-angiogenic effect of transretinoic acid. Retinoic acid, as well as derivatives thereof which may also be utilized in the context of the present invention, may be readily obtained from commercial sources, 10 including for example, Sigma Chemical Co. (# R2625).

Taxol is a highly derivatized diterpenoid (Wani et al., *J. Am. Chem. Soc.* 93:2325, 1971) which has been obtained from the harvested and dried bark of *Taxus brevifolia* (Pacific Yew.) and *Taxomyces Andreanae* and *Endophytic Fungus* of the Pacific Yew. (Stierle et al., *Science* 60:214-216, 1993). 15 Generally, taxol acts to stabilize microtubular structures by binding tubulin to form abnormal mitotic spindles. "Taxol" (which should be understood herein to include analogues and derivatives of taxol such as, for example, baccatin and taxotere) may be readily prepared utilizing techniques known to those skilled in the art (see also WO 94/07882, WO 94/07881, WO 94/07880, WO 94/07876, 20 WO 93/23555, WO 93/10076, U.S. Patent Nos. 5,294,637, 5,283,253, 5,279,949, 5,274,137, 5,202,448, 5,200,534, 5,229,526, and EP 590267) or obtained from a variety of commercial sources, including for example, Sigma Chemical Co., St. Louis, Missouri (T7402 - from *Taxus brevifolia*).

Suramin is a polysulfonated naphthylurea compound that is 25 typically used as a trypanocidal agent. Briefly, Suramin blocks the specific cell surface binding of various growth factors such as platelet derived growth factor ("PDGF"), epidermal growth factor ("EGF"), transforming growth factor ("TGF- β "), insulin-like growth factor ("IGF-1") and fibroblast growth factor (" β FGF"). Suramin may be prepared in accordance with known techniques, or readily 30 obtained from a variety of commercial sources, including for example Mobay Chemical Co., New York. (see Gagliardi et al., *Cancer Res.* 52:5073-5075, 1992; and Coffey, Jr., et al., *J. of Cell. Phys.* 132:143-148, 1987).

Tissue Inhibitor of Metalloproteinases-1 ("TIMP") is secreted by endothelial cells which also secrete MTPases. TIMP is glycosylated and has a 35 molecular weight of 28.5 kDa. TIMP-1 regulates angiogenesis by binding to activated metalloproteinases, thereby suppressing the invasion of blood vessels into the extracellular matrix. Tissue Inhibitor of Metalloproteinases-2 ("TIMP-

2") may also be utilized to inhibit angiogenesis. Briefly, TIMP-2 is a 21 kDa nonglycosylated protein which binds to metalloproteinases in both the active and latent, proenzyme forms. Both TIMP-1 and TIMP-2 may be obtained from commercial sources such as Synergen, Boulder, Colorado.

5 Plasminogen Activator Inhibitor - 1 (PAI-1) is a 50 kDa glycoprotein which is present in blood platelets, and can also be synthesized by endothelial cells and muscle cells. PAI-1 inhibits t-PA and urokinase plasminogen activator at the basolateral site of the endothelium, and additionally regulates the fibrinolysis process. Plasminogen Activator Inhibitor-2 (PAI-2) is generally
10 found only in the blood under certain circumstances such as in pregnancy, and in the presence of tumors. Briefly, PAI-2 is a 56 kDa protein which is secreted by monocytes and macrophages. It is believed to regulate fibrinolytic activity, and in particular inhibits urokinase plasminogen activator and tissue plasminogen activator, thereby preventing fibrinolysis.

15 A wide variety of other anti-angiogenic factors may also be utilized within the context of the present invention. Representative examples include Platelet Factor 4 (Sigma Chemical Co., #F1385); Protamine Sulphate (Clupeine) (Sigma Chemical Co., #P4505); Sulphated Chitin Derivatives (prepared from queen crab shells), (Sigma Chemical Co., #C3641; Murata
20 et al., *Cancer Res.* 51:22-26, 1991); Sulphated Polysaccharide Peptidoglycan Complex (SP-PG) (the function of this compound may be enhanced by the presence of steroids such as estrogen and tamoxifen citrate); Staurosporine (Sigma Chemical Co., #S4400); Modulators of Matrix Metabolism, including for example, proline analogs {[(L-azetidine-2-carboxylic acid (LACA) (Sigma
25 Chemical Co., #A0760)), cishydroxyproline, d,L-3,4-dehydroproline (Sigma Chemical Co., #D0265), Thiaproline (Sigma Chemical Co., #T0631)], α,α -dipyridyl (Sigma Chemical Co., #D7505), β -aminopropionitrile fumarate (Sigma Chemical Co., #A3134)]}; MDL 27032 (4-propyl-5-(4-pyridinyl)-2(3H)-oxazolone; Merion Merrel Dow Research Institute); Methotrexate (Sigma
30 Chemical Co., #A6770; Hirata et al., *Arthritis and Rheumatism* 32:1065-1073, 1989); Mitoxantrone (Polverini and Novak, *Biochem. Biophys. Res. Comm.* 140:901-907); Heparin (Folkman, *Bio. Phar.* 34:905-909, 1985; Sigma Chemical Co., #P8754); Interferons (e.g., Sigma Chemical Co., #13265); 2 Macroglobulin-serum (Sigma Chemical Co., #M7151); ChIMP-3 (Pavloff et al., *J. Bio. Chem.*
35 267:17321-17326, 1992); Chymostatin (Sigma Chemical Co., #C7268; Tomkinson et al., *Biochem J.* 286:475-480, 1992); β -Cyclodextrin Tetradasulfate (Sigma Chemical Co., #C4767); Eponemycin; Estramustine

- (available from Sigma; Wang and Stearns *Cancer Res.* 48:6262-6271, 1988); Fumagillin (Sigma Chemical Co., #F6771; Canadian Patent No. 2,024,306; Ingber et al., *Nature* 348:555-557, 1990); Gold Sodium Thiomalate ("GST"; Sigma:G4022; Matsubara and Ziff, *J. Clin. Invest.* 79:1440-1446, 1987); (D-
5 Penicillamine ("CDPT"; Sigma Chemical Co., #P4875 or P5000(HCl)); β -1-
anticollagenase-serum; α 2-antiplasmin (Sigma Chem. Co.:A0914; Holmes et al.,
J. Biol. Chem. 262(4):1659-1664, 1987); Bisantrone (National Cancer Institute);
Lobenzarit disodium (N-(2)-carboxyphenyl-4-chloroanthronilic acid disodium or
"CCA"; Takeuchi et al., *Agents Actions* 36:312-316, 1992); Thalidomide,
10 Angiostatic steroid, AGM-1470, carboxyaminolmidazole, metalloproteinase
inhibitors such as BB94 and the peptide CDPGYIGSR-NH₂ (SEQUENCE ID
NO. 1) (Iwaki Glass, Tokyo, Japan).

Anti-angiogenic compositions of the present invention may additionally comprise a wide variety of compounds in addition to the anti-
15 angiogenic factor and polymeric carrier. For example, anti-angiogenic
compositions of the present invention may also, within certain embodiments of
the invention, also comprise one or more antibiotics, anti-inflammatories, anti-
viral agents, anti-fungal agents and/or anti-protozoal agents. Representative
examples of antibiotics included within the compositions described herein
20 include: penicillins; cephalosporins such as cefadroxil, cefazolin, cefaclor;
aminoglycosides such as gentamycin and tobramycin; sulfonamides such as
sulfamethoxazole; and metronidazole. Representative examples of anti-
inflammatories include: steroids such as prednisone, prednisolone,
hydrocortisone, adrenocorticotrophic hormone, and sulfasalazine; and non-
25 steroidal anti-inflammatory drugs ("NSAIDS") such as aspirin, ibuprofen,
naproxen, fenopofen, indomethacin, and phenylbutazone. Representative
examples of antiviral agents include acyclovir, ganciclovir, zidovudine.
Representative examples of antifungal agents include: nystatin, ketoconazole,
griseofulvin, flucytosine, miconazole, clotrimazole. Representative examples of
30 antiprotozoal agents include: pentamidine isethionate, quinine, chloroquine,
and mefloquine.

Anti-angiogenic compositions of the present invention may also contain one or more hormones such as thyroid hormone, estrogen,
progesterone, cortisone and/or growth hormone, other biologically active
35 molecules such as insulin, as well as T_H1 (e.g., Interleukins -2, -12, and -15,
gamma interferon or T_H2 (e.g., Interleukins -4 and -10) cytokines.

Anti-angiogenic compositions of the present invention may also comprise additional ingredients such as surfactants (either hydrophilic or hydrophobic; see Example 13), anti-neoplastic or chemotherapeutic agents (e.g., 5-fluorouracil, vinblastine, doxyrubicin, adriamycin, or tamocifen), radioactive
5 agents (e.g., Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212) or toxins (e.g., ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A).

As noted above, anti-angiogenic compositions of the present
10 invention comprise an anti-angiogenic factor and a polymeric carrier. In addition to the wide array of anti-angiogenic factors and other compounds discussed above, anti-angiogenic compositions of the present invention may include a wide variety of polymeric carriers, including for example both biodegradable and non-biodegradable compositions. Representative examples
15 of biodegradable compositions include albumin, gelatin, starch, cellulose, destrans, polysaccharides, fibrinogen, poly (d,l lactide), poly (d,l-lactide-co-glycolide), poly (glycolide), poly (hydroxybutyrate), poly (alkylcarbonate) and poly (orthoesters) (see generally, Illum, L., Davids, S.S. (eds.) "Polymers in controlled Drug Delivery" Wright, Bristol, 1987; Arshady, J. *Controlled Release*
20 17:1-22, 1991; Pitt, *Int. J. Phar.* 59:173-196, 1990; Holland et al., *J. Controlled Release* 4:155-0180, 1986). Representative examples of nondegradable polymers include EVA copolymers, silicone rubber and poly (methylmethacrylate). Particularly preferred polymeric carriers include EVA copolymer (e.g., ELVAX 40, poly(ethylene-vinyl acetate) crosslinked with 40% vinyl acetate; DuPont),
25 poly(lactic-co-glycolic acid), polycaprolactone, polylactic acid, copolymers of poly(ethylene-vinyl acetate) crosslinked with 40% vinyl acetate and polylactic acid, and copolymers of polylactic acid and polycaprolactone.

Polymeric carriers may be fashioned in a variety of forms, including for example, as nanospheres or microspheres, rod-shaped devices,
30 pellets, slabs, or capsules (see, e.g., Goodell et al., *Am. J. Hosp. Pharm.* 43:1454-1461, 1986; Langer et al., "Controlled release of macromolecules from polymers", in *Biomedical polymers, Polymeric materials and pharmaceuticals for biomedical use*, Goldberg, E.P., Nakagim, A. (eds.) Academic Press, pp. 113-137, 1980; Rhine et al., *J. Pharm. Sci.* 69:265-270, 1980; Brown et al., *J. Pharm. Sci.*
35 72:1181-1185, 1983; and Bawa et al., *J. Controlled Release* 1:259-267, 1985).

Preferably, anti-angiogenic compositions of the present invention (which comprise one or more anti-angiogenic factors, and a polymeric carrier)

are fashioned in a manner appropriate to the intended use. Within preferred aspects of the present invention, the anti-angiogenic composition should be biocompatible, and release one or more anti-angiogenic factors over a period of several weeks to months. In addition, anti-angiogenic compositions of the present invention should preferably be stable for several months and capable of being produced and maintained under sterile conditions.

Within certain aspects of the present invention, anti-angiogenic compositions may be fashioned in any size ranging from nanospheres to microspheres (*e.g.*, from 0.1 μm to 500 μm), depending upon the particular use. For example, when used for the purpose of tumor embolization (as discussed below), it is generally preferable to fashion the anti-angiogenic composition in microspheres of between 15 and 500 μm , preferably between 15 and 200 μm , and most preferably, between 25 and 150 μm . Such nanoparticles may also be readily applied as a "spray", which solidifies into a film or coating. Nanoparticles (also termed "nanospheres") may be prepared in a wide array of sizes, including for example, from 0.1 μm to 3 μm , from 10 μm to 30 μm , and from 30 μm to 100 μm (see Example 8).

Anti-angiogenic compositions may also be prepared, given the disclosure provided herein, for a variety of other applications. For example, for the administration of anti-angiogenic compositions to the cornea, the compositions of the present invention may be incorporated into polymers as nanoparticles (see generally, Kreuter *J. Controlled Release* 16:169-176, 1991; Couvreur and Vauthier, *J. Controlled Release* 17:187-198, 1991). Such nanoparticles may also be readily applied as a "spray", which solidifies into a film or coating. Nanoparticles (also termed "nanospheres") may be prepared in a wide array of sizes, including for example, from 0.1 μm to 3 μm , from 10 μm to 30 μm , and from 30 μm to 100 μm (see Example 8).

Anti-angiogenic compositions of the present invention may also be prepared in a variety of "paste" or gel forms. For example, within one embodiment of the invention, anti-angiogenic compositions are provided which are liquid at one temperature (*e.g.*, temperature greater than 37°C, such as 40°C, 45°C, 50°C, 55°C or 60°C), and solid or semi-solid at another temperature (*e.g.*, ambient body temperature, or any temperature lower than 37°C). Such "thermopastes" may be readily made given the disclosure provided herein (see, *e.g.*, Examples 10 and 14).

Within yet other aspects of the invention, the anti-angiogenic compositions of the present invention may be formed as a film. Preferably, such

films are generally less than 5, 4, 3, 2, or 1, mm thick, more preferably less than 0.75 mm or 0.5 mm thick, and most preferably less than 500 μm to 100 μm thick. Such films are preferably flexible with a good tensile strength (*e.g.*, greater than 50, preferably greater than 100, and more preferably greater than 150 or 200 N/cm²), good adhesive properties (*i.e.*, readily adheres to moist or wet surfaces), and has controlled permeability. Representative examples of such films are set forth below in the Examples (*see e.g.*, Example 13).

Representative examples of the incorporation of anti-angiogenic factors such as into a polymeric carriers are described in more detail below in Examples 3, 4 and 8-15.

ARTERIAL EMBOLIZATION

In addition to the compositions described above, the present invention also provides a variety of methods which utilize the above-described anti-angiogenic compositions. In particular, within one aspect of the present invention methods are provided for embolizing a blood vessel, comprising the step of delivering into the vessel a therapeutically effective amount of an anti-angiogenic composition (as described above), such that the blood vessel is effectively occluded. Therapeutically effective amounts suitable for occluding blood vessels may be readily determined given the disclosure provided below, and as described in Example 6. Within a particularly preferred embodiment, the anti-angiogenic composition is delivered to a blood vessel which nourishes a tumor (see Figure 13).

Briefly, there are a number of clinical situations (*e.g.*, bleeding, tumor development) where it is desirable to reduce or abolish the blood supply to an organ or region. As described in greater detail below, this may be accomplished by injecting anti-angiogenic compositions of the present invention into a desired blood vessel through a selectively positioned catheter (see Figure 13). The composition travels via the blood stream until it becomes wedged in the vasculature, thereby physically (or chemically) occluding the blood vessel. The reduced or abolished blood flow to the selected area results in infarction (cell death due to an inadequate supply of oxygen and nutrients) or reduced blood loss from a damaged vessel.

For use in embolization therapy, anti-angiogenic compositions of the present invention are preferably non-toxic, thrombogenic, easy to inject down vascular catheters, radio-opaque, rapid and permanent in effect, sterile, and readily available in different shapes or sizes at the time of the procedure.

In addition, the compositions preferably result in the slow (ideally, over a period of several weeks to months) release of an anti-angiogenic factor. Particularly preferred anti-angiogenic compositions should have a predictable size of 15-200 μm after being injected into the vascular system. Preferably, they should not clump into larger particles either in solution or once injected. In addition, preferable compositions should not change shape or physical properties during storage prior to use.

Embolization therapy may be utilized in at least three principal ways to assist in the management of neoplasms: (1) definitive treatment of tumors (usually benign); (2) for preoperative embolization; and (3) for palliative embolization. Briefly, benign tumors may sometimes be successfully treated by embolization therapy alone. Examples of such tumors include simple tumors of vascular origin (*e.g.*, haemangiomas), endocrine tumors such as parathyroid adenomas, and benign bone tumors.

For other tumors, (*e.g.*, renal adenocarcinoma), preoperative embolization may be employed hours or days before surgical resection in order to reduce operative blood loss, shorten the duration of the operation, and reduce the risk of dissemination of viable malignant cells by surgical manipulation of the tumor. Many tumors may be successfully embolized preoperatively, including for example nasopharyngeal tumors, glomus jugular tumors, meningiomas, chemodectomas, and vagal neuromas.

Embolization may also be utilized as a primary mode of treatment for inoperable malignancies, in order to extend the survival time of patients with advanced disease. Embolization may produce a marked improvement in the quality of life of patients with malignant tumors by alleviating unpleasant symptoms such as bleeding, venous obstruction and tracheal compression. The greatest benefit from palliative tumor embolization, however, may be seen in patients suffering from the humoral effects of malignant endocrine tumors, wherein metastases from carcinoid tumors and other endocrine neoplasms such as insulinomas and glucagonomas may be slow growing, and yet cause great distress by virtue of the endocrine syndromes which they produce.

In general, embolization therapy utilizing anti-angiogenic compositions of the present invention is typically performed in a similar manner, regardless of the site. Briefly, angiography (a road map of the blood vessels) of the area to be embolized is first performed by injecting radiopaque contrast through a catheter inserted into an artery or vein (depending on the site to be embolized) as an X-ray is taken. The catheter may be inserted either

percutaneously or by surgery. The blood vessel is then embolized by refluxing anti-angiogenic compositions of the present invention through the catheter, until flow is observed to cease. Occlusion may be confirmed by repeating the angiogram.

5 Embolization therapy generally results in the distribution of compositions containing anti-angiogenic factors throughout the interstices of the tumor or vascular mass to be treated. The physical bulk of the embolic particles clogging the arterial lumen results in the occlusion of the blood supply. In addition to this effect, the presence of an anti-angiogenic factor(s) prevents the
10 formation of new blood vessels to supply the tumor or vascular mass, enhancing the devitalizing effect of cutting off the blood supply.

 Therefore, it should be evident that a wide variety of tumors may be embolized utilizing the compositions of the present invention. Briefly, tumors are typically divided into two classes: benign and malignant. In a benign
15 tumor the cells retain their differentiated features and do not divide in a completely uncontrolled manner. In addition, the tumor is localized and nonmetastatic. In a malignant tumor, the cells become undifferentiated, do not respond to the body's growth and hormonal signals, and multiply in an uncontrolled manner; the tumor is invasive and capable of spreading to distant
20 sites (metastasizing).

 Within one aspect of the present invention, metastases (secondary tumors) of the liver may be treated utilizing embolization therapy. Briefly, a catheter is inserted via the femoral or brachial artery and advanced into the hepatic artery by steering it through the arterial system under fluoroscopic
25 guidance. The catheter is advanced into the hepatic arterial tree as far as necessary to allow complete blockage of the blood vessels supplying the tumor(s), while sparing as many of the arterial branches supplying normal structures as possible. Ideally this will be a segmental branch of the hepatic artery, but it could be that the entire hepatic artery distal to the origin of the
30 gastroduodenal artery, or even multiple separate arteries, will need to be blocked depending on the extent of tumor and its individual blood supply. Once the desired catheter position is achieved, the artery is embolized by injecting anti-angiogenic compositions (as described above) through the arterial catheter until flow in the artery to be blocked ceases, preferably even after observation
35 for 5 minutes. Occlusion of the artery may be confirmed by injecting radiopaque contrast through the catheter and demonstrating by fluoroscopy or

X-ray film that the vessel which previously filled with contrast no longer does so. The same procedure may be repeated with each feeding artery to be occluded.

As noted above, both benign and malignant tumors may be embolized utilizing compositions of the present invention. Representative
5 examples of benign hepatic tumors include Hepatocellular Adenoma, Cavernous Haemangioma, and Focal Nodular Hyperplasia. Other benign tumors, which are more rare and often do not have clinical manifestations, may also be treated. These include Bile Duct Adenomas, Bile Duct Cystadenomas, Fibromas, Lipomas, Leiomyomas, Mesotheliomas, Teratomas, Myxomas, and
10 Nodular Regenerative Hyperplasia.

Malignant Hepatic Tumors are generally subdivided into two categories: primary and secondary. Primary tumors arise directly from the tissue in which they are found. Thus, a primary liver tumor is derived originally from the cells which make up the liver tissue (such as hepatocytes and biliary cells).
15 Representative examples of primary hepatic malignancies which may be treated by arterial embolization include Hepatocellularcarcinoma, Cholangiocarcinoma, Angiosarcoma, Cystadenocar-cinoma, Squamous Cell Carcinoma, and Hepatoblastoma.

A secondary tumor, or metastasis, is a tumor which originated
20 elsewhere in the body but has now spread to a distant organ. The common routes for metastasis are direct growth into adjacent structures, spread through the vascular or lymphatic systems, and tracking along tissue planes and body spaces (peritoneal fluid, cerebrospinal fluid, etc.). Secondary hepatic tumors are one of the most common causes of death in cancer patients and are by far
25 and away the most common form of liver tumor. Although virtually any malignancy can metastasize to the liver, tumors which are most likely to spread to the liver include: cancer of the stomach, colon, and pancreas; melanoma; tumors of the lung, oropharynx, and bladder; Hodgkin's and non-Hodgkin's lymphoma; tumors of the breast, ovary, and prostate. Each one of the above-
30 named primary tumors has numerous different tumor types which may be treated by arterial embolization (for example, there are over 32 different types of ovarian cancer).

As noted above, embolization therapy utilizing anti-angiogenic compositions of the present invention may also be applied to a variety of other
35 clinical situations where it is desired to occlude blood vessels. Within one aspect of the present invention, arteriovenous malformation may be treated by administration of one of the above-described compositions. Briefly,

arteriovenous malformations (vascular malformations) refers to a group of diseases wherein at least one (and most typically, many) abnormal communications between arteries and veins occur, resulting in a local tumor-like mass composed predominantly of blood vessels. Such disease may be either
5 congenital or acquired.

Within one embodiment of the invention, an arteriovenous malformation may be treated by inserting a catheter via the femoral or brachial artery, and advancing it into the feeding artery under fluoroscopic guidance. The catheter is preferably advanced as far as necessary to allow complete
10 blockage of the blood vessels supplying the vascular malformation, while sparing as many of the arterial branches supplying normal structures as possible (ideally this will be a single artery, but most often multiple separate arteries may need to be occluded, depending on the extent of the vascular malformation and its individual blood supply). Once the desired catheter position is achieved, each
15 artery may be embolized utilizing anti-angiogenic compositions of the present invention.

Within another aspect of the invention, embolization may be accomplished in order to treat conditions of excessive bleeding. For example, menorrhagia (excessive bleeding with menstruation) may be readily treated by
20 embolization of uterine arteries. Briefly, the uterine arteries are branches of the internal iliac arteries bilaterally. Within one embodiment of the invention, a catheter may be inserted via the femoral or brachial artery, and advanced into each uterine artery by steering it through the arterial system under fluoroscopic guidance. The catheter should be advanced as far as necessary to allow
25 complete blockage of the blood vessels to the uterus, while sparing as many arterial branches that arise from the uterine artery and supply normal structures as possible. Ideally a single uterine artery on each side may be embolized, but occasionally multiple separate arteries may need to be blocked depending on the individual blood supply. Once the desired catheter position is achieved,
30 each artery may be embolized by administration of the anti-angiogenic compositions as described above.

In a like manner, arterial embolization may be accomplished in a variety of other conditions, including for example for acute bleeding, vascular abnormalities, central nervous system disorders, and hypersplenism.

35

USE OF ANTI-ANGIOGENIC COMPOSITIONS AS COATINGS FOR STENTS

As noted above, the present invention also provides stents, comprising a generally tubular structure (which includes for example, spiral shapes), the surface of which is coated with a composition as described above.

5 Briefly, a stent is a scaffolding, usually cylindrical in shape, that may be inserted into a body passageway (*e.g.*, bile ducts), which has been narrowed by a disease process (*e.g.*, ingrowth by a tumor) in order to prevent closure or reclosure of the passageway. Stents act by physically holding open the walls of the body passage into which they are inserted.

10 A variety of stents may be utilized within the context of the present invention, including for example, esophageal stents, vascular stents, biliary stents, pancreatic stents, ureteric and urethral stents, lacrimal stents, eustachian tube stents, fallopian tube stents, and tracheal/bronchial stents.

15 Stents may be readily obtained from commercial sources, or constructed in accordance with well known techniques. Representative examples of stents include those described in U.S. Patent No. 4,776,337, entitled "Expandable Intraluminal Graft, and Method and Apparatus for Implanting and Expandable Intraluminal Graft", U.S. Patent No. 5,176,626, entitled "Indwelling Stent", U.S. Patent No. 5,147,370 entitled "Nitinol Stent for Hollow Body
20 Conduits", U.S. Patent No. 5,064,435 entitled "Self-Expanding Prosthesis Having Stable Axial Length", U.S. Patent No. 5,052,998 entitled "Indwelling Stent and Method of Use", and U.S. Patent No. 5,041,126 entitled "Endovascular Stent and Delivery System, all of which are hereby incorporated by reference in their entirety.

25 Stents may be coated with anti-angiogenic compositions or anti-angiogenic factors of the present invention using a variety of methods, including for example: (a) by directly affixing to the stent an anti-angiogenic composition (*e.g.*, by either spraying the stent with a polymer/drug film, or by dipping the stent into a polymer/drug solution), (b) by coating the stent with a substance
30 such as a hydrogel which will in turn absorb the anti-angiogenic composition (or anti-angiogenic factor above), (c) by interweaving anti-angiogenic composition coated thread (or the polymer itself formed into a thread) into the stent structure, (d) by inserting the stent into a sleeve or mesh which is comprised of or coated with an anti-angiogenic composition, or (e) constructing the stent
35 itself with an anti-angiogenic composition. Within preferred embodiments of the invention, the composition should firmly adhere to the stent during storage and at the time of insertion, and should not be dislodged from the stent when

the diameter is expanded from its collapsed size to its full expansion size. The anti-angiogenic composition should also preferably not degrade during storage, prior to insertion, or when warmed to body temperature after expansion inside the body. In addition, it should preferably coat the stent smoothly and evenly, with a uniform distribution of angiogenesis inhibitor, while not changing the stent contour. Within preferred embodiments of the invention, the anti-angiogenic composition should provide a uniform, predictable, prolonged release of the anti-angiogenic factor into the tissue surrounding the stent once it has been deployed. For vascular stents, in addition to the above properties, the composition should not render the stent thrombogenic (causing blood clots to form), or cause significant turbulence in blood flow (more than the stent itself would be expected to cause if it was uncoated).

Within another aspect of the present invention, methods are provided for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition (or, an anti-angiogenic factor alone), such that the passageway is expanded. A variety of embodiments are described below wherein the lumen of a body passageway is expanded in order to eliminate a biliary, esophageal, tracheal/bronchial, urethral or vascular obstruction. In addition, a representative example is described in more detail below in Example 7.

Generally, stents are inserted in a similar fashion regardless of the site or the disease being treated. Briefly, a preinsertion examination, usually a diagnostic imaging procedure, endoscopy, or direct visualization at the time of surgery, is generally first performed in order to determine the appropriate positioning for stent insertion. A guidewire is then advanced through the lesion or proposed site of insertion, and over this is passed a delivery catheter which allows a stent in its collapsed form to be inserted. Typically, stents are capable of being compressed, so that they can be inserted through tiny cavities via small catheters, and then expanded to a larger diameter once they are at the desired location. Once expanded, the stent physically forces the walls of the passageway apart and holds it open. As such, they are capable of insertion via a small opening, and yet are still able to hold open a large diameter cavity or passageway. The stent may be self-expanding (*e.g.*, the Wallstent and Gianturco stents), balloon expandable (*e.g.*, the Palmaz stent and Strecker stent), or implanted by a change in temperature (*e.g.*, the Nitinol stent).

Stents are typically maneuvered into place under radiologic or direct visual control, taking particular care to place the stent precisely across the narrowing in the organ being treated. The delivery catheter is then removed, leaving the stent standing on its own as a scaffold. A post insertion examination,
5 usually an x-ray, is often utilized to confirm appropriate positioning.

Within a preferred embodiment of the invention, methods are provided for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway, the stent having a generally tubular structure, the surface of the structure being coated with a composition as described above,
10 such that the biliary obstruction is eliminated. Briefly, tumor overgrowth of the common bile duct results in progressive cholestatic jaundice which is incompatible with life. Generally, the biliary system which drains bile from the liver into the duodenum is most often obstructed by (1) a tumor composed of bile duct cells (cholangiocarcinoma), (2) a tumor which invades the bile duct
15 (*e.g.*, pancreatic carcinoma), or (3) a tumor which exerts extrinsic pressure and compresses the bile duct (*e.g.*, enlarged lymph nodes).

Both primary biliary tumors, as well as other tumors which cause compression of the biliary tree may be treated utilizing the stents described herein. One example of primary biliary tumors are adenocarcinomas (which are
20 also called Klatskin tumors when found at the bifurcation of the common hepatic duct). These tumors are also referred to as biliary carcinomas, choledocholangiocarcinomas, or adenocarcinomas of the biliary system. Benign tumors which affect the bile duct (*e.g.*, adenoma of the biliary system), and, in rare cases, squamous cell carcinomas of the bile duct and adenocarcinomas of
25 the gallbladder, may also cause compression of the biliary tree, and therefore, result in biliary obstruction.

Compression of the biliary tree is most commonly due to tumors of the liver and pancreas which compress and therefore obstruct the ducts. Most of the tumors from the pancreas arise from cells of the pancreatic ducts.
30 This is a highly fatal form of cancer (5% of all cancer deaths; 26,000 new cases per year in the U.S.) with an average of 6 months survival and a 1 year survival rate of only 10%. When these tumors are located in the head of the pancreas they frequently cause biliary obstruction, and this detracts significantly from the quality of life of the patient. While all types of pancreatic tumors are generally
35 referred to as "carcinoma of the pancreas," there are histologic subtypes including: adenocarcinoma, adenosquamous carcinoma, cystadeno-carcinoma, and acinar cell carcinoma. Hepatic tumors, as discussed above, may also cause

compression of the biliary tree, and therefore cause obstruction of the biliary ducts.

Within one embodiment of the invention, a biliary stent is first inserted into a biliary passageway in one of several ways: from the top end by inserting a needle through the abdominal wall and through the liver (a percutaneous transhepatic cholangiogram or "PTC"); from the bottom end by cannulating the bile duct through an endoscope inserted through the mouth, stomach, or duodenum (an endoscopic retrograde cholangiogram or "ERCP"); or by direct incision during a surgical procedure. A preinsertion examination, PTC, ERCP, or direct visualization at the time of surgery should generally be performed to determine the appropriate position for stent insertion. A guidewire is then advanced through the lesion, and over this a delivery catheter is passed to allow the stent to be inserted in its collapsed form. If the diagnostic exam was a PTC, the guidewire and delivery catheter will be inserted via the abdominal wall, while if the original exam was an ERCP the stent will be placed via the mouth. The stent is then positioned under radiologic, endoscopic, or direct visual control taking particular care to place it precisely across the narrowing in the bile duct. The delivery catheter will be removed leaving the stent standing as a scaffolding which holds the bile duct open. A further cholangiogram will be performed to document that the stent is appropriately positioned.

Within yet another embodiment of the invention, methods are provided for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the esophageal obstruction is eliminated. Briefly, the esophagus is the hollow tube which transports food and liquids from the mouth to the stomach. Cancer of the esophagus or invasion by cancer arising in adjacent organs (*e.g.*, cancer of the stomach or lung) results in the inability to swallow food or saliva. Within this embodiment, a preinsertion examination, usually a barium swallow or endoscopy should generally be performed in order to determine the appropriate position for stent insertion. A catheter or endoscope may then be positioned through the mouth, and a guidewire is advanced through the blockage. A stent delivery catheter is passed over the guidewire under radiologic or endoscopic control, and a stent is placed precisely across the narrowing in the esophagus. A post insertion examination,

usually a barium swallow x-ray, may be utilized to confirm appropriate positioning.

Within other embodiments of the invention, methods are provided for eliminating tracheal/bronchial obstructions, comprising inserting a
5 tracheal/bronchial stent into the trachea or bronchi, the stent having a generally tubular structure, the surface of which is coated with an anti-angiogenic composition as described above, such that the tracheal/bronchial obstruction is eliminated. Briefly, the trachea and bronchi are tubes which carry air from the
10 mouth and nose to the lungs. Blockage of the trachea by cancer, invasion by cancer arising in adjacent organs (*e.g.*, cancer of the lung), or collapse of the trachea or bronchi due to chondromalacia (weakening of the cartilage rings) results in inability to breathe. Within this embodiment of the invention, preinsertion examination, usually an endoscopy, should generally be performed in order to determine the appropriate position for stent insertion. A catheter or
15 endoscope is then positioned through the mouth, and a guidewire advanced through the blockage. A delivery catheter is then passed over the guidewire in order to allow a collapsed stent to be inserted. The stent is placed under radiologic or endoscopic control in order to place it precisely across the narrowing. The delivery catheter may then be removed leaving the stent
20 standing as a scaffold on its own. A post insertion examination, usually a bronchoscopy, may be utilized to confirm appropriate positioning.

Within another embodiment of the invention, methods are provided for eliminating urethral obstructions, comprising inserting a urethral
25 stent into a urethra, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the urethral obstruction is eliminated. Briefly, the urethra is the tube which drains the bladder through the penis. Extrinsic narrowing of the urethra as it passes through the prostate, due to hypertrophy of the prostate, occurs in virtually every man over the age of 60 and causes progressive difficulty
30 with urination. Within this embodiment, a preinsertion examination, usually an endoscopy or urethrogram should generally first be performed in order to determine the appropriate position for stent insertion, which is above the external urinary sphincter at the lower end, and close to flush with the bladder neck at the upper end. An endoscope or catheter is then positioned through the
35 penile opening and a guidewire advanced into the bladder. A delivery catheter is then passed over the guidewire in order to allow stent insertion. The delivery catheter is then removed, and the stent expanded into place. A post insertion

examination, usually endoscopy or retrograde urethrogram, may be utilized to confirm appropriate position.

Within another embodiment of the invention, methods are provided for eliminating vascular obstructions, comprising inserting a vascular
5 stent into a blood vessel, the stent having a generally tubular structure, the surface of the structure being coated with an anti-angiogenic composition as described above, such that the vascular obstruction is eliminated. Briefly, stents may be placed in a wide array of blood vessels, both arteries and veins, to prevent recurrent stenosis at the site of failed angioplasties, to treat narrowings
10 that would likely fail if treated with angioplasty, and to treat post surgical narrowings (*e.g.*, dialysis graft stenosis). Representative examples of suitable sites include the iliac, renal, and coronary arteries, the superior vena cava, and in dialysis grafts. Within one embodiment, angiography is first performed in order to localize the site for placement of the stent. This is typically
15 accomplished by injecting radiopaque contrast through a catheter inserted into an artery or vein as an x-ray is taken. A catheter may then be inserted either percutaneously or by surgery into the femoral artery, brachial artery, femoral vein, or brachial vein, and advanced into the appropriate blood vessel by steering it through the vascular system under fluoroscopic guidance. A stent
20 may then be positioned across the vascular stenosis. A post insertion angiogram may also be utilized in order to confirm appropriate positioning.

USE OF ANTI-ANGIOGENIC COMPOSITIONS IN SURGICAL PROCEDURES

As noted above, anti-angiogenic compositions may be utilized in a
25 wide variety of surgical procedures. For example, within one aspect of the present invention an anti-angiogenic compositions (in the form of, for example, a spray or film) may be utilized to coat or spray an area prior to removal of a tumor, in order to isolate normal surrounding tissues from malignant tissue, and/or to prevent the spread of disease to surrounding tissues. Within other
30 aspects of the present invention, anti-angiogenic compositions (*e.g.*, in the form of a spray) may be delivered via endoscopic procedures in order to coat tumors, or inhibit angiogenesis in a desired locale. Within yet other aspects of the present invention, surgical meshes which have been coated with anti-angiogenic compositions of the present invention may be utilized in any procedure wherein
35 a surgical mesh might be utilized. For example, within one embodiment of the invention a surgical mesh ladened with an anti-angiogenic composition may be utilized during abdominal cancer resection surgery (*e.g.*, subsequent to colon

resection) in order to provide support to the structure, and to release an amount of the anti-angiogenic factor.

Within further aspects of the present invention, methods are provided for treating tumor excision sites, comprising administering an anti-angiogenic composition as described above to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within one embodiment of the invention, the anti-angiogenic composition(s) (or anti-angiogenic factor(s) alone) are administered directly to the tumor excision site (*e.g.*, applied by swabbing, brushing or otherwise coating the resection margins of the tumor with the anti-angiogenic composition(s) or factor(s)). Alternatively, the anti-angiogenic composition(s) or factor(s) may be incorporated into known surgical pastes prior to administration. Within particularly preferred embodiments of the invention, the anti-angiogenic compositions are applied after hepatic resections for malignancy, and after neurosurgical operations.

Within one aspect of the present invention, anti-angiogenic compositions (as described above) may be administered to the resection margin of a wide variety of tumors, including for example, breast, colon, brain and hepatic tumors. For example, within one embodiment of the invention anti-angiogenic compositions may be administered to the site of a neurological tumor subsequent to excision, such that the formation of new blood vessels at the site are inhibited. Briefly, the brain is highly functionally localized; *i.e.*, each specific anatomical region is specialized to carry out a specific function. Therefore it is the location of brain pathology that is often more important than the type. A relatively small lesion in a key area can be far more devastating than a much larger lesion in a less important area. Similarly, a lesion on the surface of the brain may be easy to resect surgically, while the same tumor located deep in the brain may not (one would have to cut through too many vital structures to reach it). Also, even benign tumors can be dangerous for several reasons: they may grow in a key area and cause significant damage; even though they would be cured by surgical resection this may not be possible; and finally, if left unchecked they can cause increased intracranial pressure. The skull is an enclosed space incapable of expansion. Therefore, if something is growing in one location, something else must be being compressed in another location - the result is increased pressure in the skull or increased intracranial pressure. If such a condition is left untreated, vital structures can be compressed, resulting in death. The incidence of CNS (central nervous system)

malignancies is 8-16 per 100,000. The prognosis of primary malignancy of the brain is dismal, with a median survival of less than one year, even following surgical resection. These tumors, especially gliomas, are predominantly a local disease which recur within 2 centimeters of the original focus of disease after
5 surgical removal.

Representative examples of brain tumors which may be treated utilizing the compositions and methods described herein include Glial Tumors (such as Anaplastic Astrocytoma, Glioblastoma Multiform, Pilocytic Astrocytoma, Oligodendroglioma, Ependymoma, Myxopapillary Ependymoma,
10 Subependymoma, Choroid Plexus Papilloma); Neuron Tumors (e.g., Neuroblastoma, Ganglioneuroblastoma, Ganglioneuroma, and Medulloblastoma); Pineal Gland Tumors (e.g., Pineoblastoma and Pineocytoma); Menigeal Tumors (e.g., Meningioma, Meningeal Hemangiopericytoma, Meningeal Sarcoma); Tumors of Nerve Sheath Cells
15 (e.g., Schwannoma (Neurolemmoma) and Neurofibroma); Lymphomas (e.g., Hodgkin's and Non-Hodgkin's Lymphoma (including numerous subtypes, both primary and secondary); Malformative Tumors (e.g., Craniopharyngioma, Epidermoid Cysts, Dermoid Cysts and Colloid Cysts); and Metastatic Tumors (which can be derived from virtually any tumor, the most common being from
20 lung, breast, melanoma, kidney, and gastrointestinal tract tumors).

OTHER THERAPEUTIC USES OF ANTI-ANGIOGENIC COMPOSITIONS

In addition to tumors, numerous other non-tumorigenic angiogenesis-dependent diseases which are characterized by the abnormal
25 growth of blood vessels may also be treated with the anti-angiogenic compositions, or anti-angiogenic factors of the present invention. Representative examples of such non-tumorigenic angiogenesis-dependent diseases include corneal neovascularization, hypertrophic scars and keloids, proliferative diabetic retinopathy, rheumatoid arthritis, arteriovenous
30 malformations (discussed above), atherosclerotic plaques, delayed wound healing, hemophilic joints, nonunion fractures, Osler-Weber syndrome, psoriasis, pyogenic granuloma, scleroderma, trachoma, menorrhagia (discussed above) and vascular adhesions.

In particular, within one aspect of the present invention methods
35 are provided for treating corneal neovascularization (including corneal graft neovascularization), comprising the step of administering a therapeutically effective amount of an anti-angiogenic composition (as described above) to the

cornea, such that the formation of blood vessels is inhibited. Briefly, the cornea is a tissue which normally lacks blood vessels. In certain pathological conditions, however, capillaries may extend into the cornea from the pericorneal vascular plexus of the limbus. When the cornea becomes vascularized, it also
5 becomes clouded, resulting in a decline in the patient's visual acuity. Visual loss may become complete if the cornea completely opacitates.

Blood vessels can enter the cornea in a variety of patterns and depths, depending upon the process which incites the neovascularization. These patterns have been traditionally defined by ophthalmologists in the following
10 types: pannus trachomatosus, pannus leprosus, pannus phyltenulosus, pannus degenerativus, and glaucomatous pannus. The corneal stroma may also be invaded by branches of the anterior ciliary artery (called interstitial vascularization) which causes several distinct clinical lesions: terminal loops, a "brush-like" pattern, an umbel form, a lattice form, interstitial arcades (from
15 episcleral vessels), and aberrant irregular vessels.

A wide variety of disorders can result in corneal neovascularization, including for example corneal infections (*e.g.*, trachoma, herpes simplex keratitis, leishmaniasis and onchocerciasis), immunological processes (*e.g.*, graft rejection and Stevens-Johnson's syndrome), alkali burns,
20 trauma, inflammation (of any cause), toxic and nutritional deficiency states, and as a complication of wearing contact lenses.

While the cause of corneal neovascularization may vary, the response of the cornea to the insult and the subsequent vascular ingrowth is similar regardless of the cause. Briefly, the location of the injury appears to be
25 of importance as only those lesions situated within a critical distance of the limbus will incite an angiogenic response. This is likely due to the fact that the angiogenic factors responsible for eliciting the vascular invasion are created at the site of the lesion, and must diffuse to the site of the nearest blood vessels (the limbus) in order to exert their effect. Past a certain distance from the
30 limbus, this would no longer be possible and the limbic endothelium would not be induced to grow into the cornea. Several angiogenic factors are likely involved in this process, many of which are products of the inflammatory response. Indeed, neovascularization of the cornea appears to only occur in association with an inflammatory cell infiltrate, and the degree of angiogenesis
35 is proportional to the extent of the inflammatory reaction. Corneal edema further facilitates blood vessel ingrowth by loosening the corneal stromal

framework and providing a pathway of "least resistance" through which the capillaries can grow.

Following the initial inflammatory reaction, capillary growth into the cornea proceeds in the same manner as it occurs in other tissues. The
5 normally quiescent endothelial cells of the limbic capillaries and venules are stimulated to divide and migrate. The endothelial cells project away from their vessels of origin, digest the surrounding basement membrane and the tissue through which they will travel, and migrate towards the source of the angiogenic stimulus. The blind ended sprouts acquire a lumen and then anastomose
10 together to form capillary loops. The end result is the establishment of a vascular plexus within the corneal stroma.

Anti-angiogenic compositions of the present invention are useful by blocking the stimulatory effects of angiogenesis promoters, reducing endothelial cell division, decreasing endothelial cell migration, and impairing
15 the activity of the proteolytic enzymes secreted by the endothelium.

Within particularly preferred embodiments of the invention, an anti-angiogenic factor may be prepared for topical administration in saline (combined with any of the preservatives and antimicrobial agents commonly used in ocular preparations), and administered in eyedrop form. The anti-
20 angiogenic factor solution may be prepared in its pure form and administered several times daily. Alternatively, anti-angiogenic compositions, prepared as described above, may also be administered directly to the cornea. Within preferred embodiments, the anti-angiogenic composition is prepared with a muco-adhesive polymer which binds to cornea. Within further embodiments,
25 the anti-angiogenic factors or anti-angiogenic compositions may be utilized as an adjunct to conventional steroid therapy.

Topical therapy may also be useful prophylactically in corneal lesions which are known to have a high probability of inducing an angiogenic response (such as chemical burns). In these instances the treatment, likely in
30 combination with steroids, may be instituted immediately to help prevent subsequent complications.

Within other embodiments, the anti-angiogenic compositions described above may be injected directly into the corneal stroma by an ophthalmologist under microscopic guidance. The preferred site of injection
35 may vary with the morphology of the individual lesion, but the goal of the administration would be to place the composition at the advancing front of the vasculature (*i.e.*, interspersed between the blood vessels and the normal cornea).

In most cases this would involve perilimbic corneal injection to "protect" the cornea from the advancing blood vessels. This method may also be utilized shortly after a corneal insult in order to prophylactically prevent corneal neovascularization. In this situation the material could be injected in the
5 perilimbic cornea interspersed between the corneal lesion and its undesired potential limbic blood supply. Such methods may also be utilized in a similar fashion to prevent capillary invasion of transplanted corneas. In a sustained-release form, injections might only be required 2-3 times per year. A steroid could also be added to the injection solution to reduce inflammation resulting
10 from the injection itself.

Within another aspect of the present invention, methods are provided for treating hypertrophic scars and keloids, comprising the step of administering one of the above-described anti-angiogenic compositions to a hypertrophic scar or keloid.

15 Briefly, healing of wounds and scar formation occurs in three phases: inflammation, proliferation, and maturation. The first phase, inflammation, occurs in response to an injury which is severe enough to break the skin. During this phase, which lasts 3 to 4 days, blood and tissue fluid form an adhesive coagulum and fibrinous network which serves to bind the wound
20 surfaces together. This is then followed by a proliferative phase in which there is ingrowth of capillaries and connective tissue from the wound edges, and closure of the skin defect. Finally, once capillary and fibroblastic proliferation has ceased, the maturation process begins wherein the scar contracts and becomes less cellular, less vascular, and appears flat and white. This final phase
25 may take between 6 and 12 months.

If too much connective tissue is produced and the wound remains persistently cellular, the scar may become red and raised. If the scar remains within the boundaries of the original wound it is referred to as a hypertrophic scar, but if it extends beyond the original scar and into the surrounding tissue,
30 the lesion is referred to as a keloid. Hypertrophic scars and keloids are produced during the second and third phases of scar formation. Several wounds are particularly prone to excessive endothelial and fibroblastic proliferation, including burns, open wounds, and infected wounds. With hypertrophic scars, some degree of maturation occurs and gradual improvement occurs. In the case
35 of keloids however, an actual tumor is produced which can become quite large. Spontaneous improvement in such cases rarely occurs.

Therefore, within one embodiment of the present invention either anti-angiogenic factors alone, or anti-angiogenic compositions as described above, are directly injected into a hypertrophic scar or keloid in order to prevent the progression of these lesions. The frequency of injections will depend upon the release kinetics of the polymer used (if present), and the clinical response. This therapy is of particular value in the prophylactic treatment of conditions which are known to result in the development of hypertrophic scars and keloids (e.g., burns), and is preferably initiated after the proliferative phase has had time to progress (approximately 14 days after the initial injury), but before hypertrophic scar or keloid development.

Within another aspect of the present invention methods are provided for treating neovascular glaucoma, comprising the step of administering a therapeutically effective amount of an anti-angiogenic composition to the eye, such that the formation of blood vessels is inhibited.

Briefly, neovascular glaucoma is a pathological condition wherein new capillaries develop in the iris of the eye. The angiogenesis usually originates from vessels located at the pupillary margin, and progresses across the root of the iris and into the trabecular meshwork. Fibroblasts and other connective tissue elements are associated with the capillary growth and a fibrovascular membrane develops which spreads across the anterior surface of the iris. Eventually this tissue reaches the anterior chamber angle where it forms synechiae. These synechiae in turn coalesce, scar, and contract to ultimately close off the anterior chamber angle. The scar formation prevents adequate drainage of aqueous humor through the angle and into the trabecular meshwork, resulting in an increase in intraocular pressure that may result in blindness.

Neovascular glaucoma generally occurs as a complication of diseases in which retinal ischemia is predominant. In particular, about one third of the patients with this disorder have diabetic retinopathy and 28% have central retinal vein occlusion. Other causes include chronic retinal detachment, end-stage glaucoma, carotid artery obstructive disease, retrolental fibroplasia, sickle-cell anemia, intraocular tumors, and carotid cavernous fistulas. In its early stages, neovascular glaucoma may be diagnosed by high magnification slitlamp biomicroscopy, where it reveals small, dilated, disorganized capillaries (which leak fluorescein) on the surface of the iris. Later gonioscopy demonstrates progressive obliteration of the anterior chamber angle by fibrovascular bands. While the anterior chamber angle is still open,

conservative therapies may be of assistance. However, once the angle closes surgical intervention is required in order to alleviate the pressure.

Therefore, within one embodiment of the invention anti-angiogenic factors (either alone or in an anti-angiogenic composition, as described above) may be administered topically to the eye in order to treat early forms of neovascular glaucoma.

Within other embodiments of the invention, anti-angiogenic compositions may be implanted by injection of the composition into the region of the anterior chamber angle. This provides a sustained localized increase of anti-angiogenic factor, and prevents blood vessel growth into the area. Implanted or injected anti-angiogenic compositions which are placed between the advancing capillaries of the iris and the anterior chamber angle can "defend" the open angle from neovascularization. As capillaries will not grow within a significant radius of the anti-angiogenic composition, patency of the angle could be maintained. Within other embodiments, the anti-angiogenic composition may also be placed in any location such that the anti-angiogenic factor is continuously released into the aqueous humor. This would increase the anti-angiogenic factor concentration within the humor, which in turn bathes the surface of the iris and its abnormal capillaries, thereby providing another mechanism by which to deliver the medication. These therapeutic modalities may also be useful prophylactically and in combination with existing treatments.

Within another aspect of the present invention, methods are provided for treating proliferative diabetic retinopathy, comprising the step of administering a therapeutically effective amount of an anti-angiogenic composition to the eyes, such that the formation of blood vessels is inhibited.

Briefly, the pathology of diabetic retinopathy is thought to be similar to that described above for neovascular glaucoma. In particular, background diabetic retinopathy is believed to convert to proliferative diabetic retinopathy under the influence of retinal hypoxia. Generally, neovascular tissue sprouts from the optic nerve (usually within 10 mm of the edge), and from the surface of the retina in regions where tissue perfusion is poor. Initially the capillaries grow between the inner limiting membrane of the retina and the posterior surface of the vitreous. Eventually, the vessels grow into the vitreous and through the inner limiting membrane. As the vitreous contracts, traction is applied to the vessels, often resulting in shearing of the vessels and blinding of the vitreous due to hemorrhage. Fibrous traction from scarring in the retina may also produce retinal detachment.

The conventional therapy of choice is panretinal photocoagulation to decrease retinal tissue, and thereby decrease retinal oxygen demands. Although initially effective, there is a high relapse rate with new lesions forming in other parts of the retina. Complications of this therapy include a decrease in
5 peripheral vision of up to 50% of patients, mechanical abrasions of the cornea, laser-induced cataract formation, acute glaucoma, and stimulation of subretinal neovascular growth (which can result in loss of vision). As a result, this procedure is performed only when several risk factors are present, and the risk-benefit ratio is clearly in favor of intervention.

10 Therefore, within particularly preferred embodiments of the invention, proliferative diabetic retinopathy may be treated by injection of an anti-angiogenic factor(s) (or anti-angiogenic composition) into the aqueous humor or the vitreous, in order to increase the local concentration of anti-angiogenic factor in the retina. Preferably, this treatment should be initiated
15 prior to the acquisition of severe disease requiring photocoagulation. Within other embodiments of the invention, arteries which feed the neovascular lesions may be embolized (utilizing anti-angiogenic compositions, as described above)

Within another aspect of the present invention, methods are provided for treating retrolental fibroblasia, comprising the step of
20 administering a therapeutically effective amount of an anti-angiogenic factor (or anti-angiogenic composition) to the eye, such that the formation of blood vessels is inhibited.

Briefly, retrolental fibroblasia is a condition occurring in premature infants who receive oxygen therapy. The peripheral retinal
25 vasculature, particularly on the temporal side, does not become fully formed until the end of fetal life. Excessive oxygen (even levels which would be physiologic at term) and the formation of oxygen free radicals are thought to be important by causing damage to the blood vessels of the immature retina. These vessels constrict, and then become structurally obliterated on exposure to
30 oxygen. As a result, the peripheral retina fails to vascularize and retinal ischemia ensues. In response to the ischemia, neovascularization is induced at the junction of the normal and the ischemic retina.

In 75% of the cases these vessels regress spontaneously. However, in the remaining 25% there is continued capillary growth, contraction
35 of the fibrovascular component, and traction on both the vessels and the retina. This results in vitreous hemorrhage and/or retinal detachment which can lead

to blindness. Neovascular angle-closure glaucoma is also a complication of this condition.

As it is often impossible to determine which cases will spontaneously resolve and which will progress in severity, conventional
5 treatment (*i.e.*, surgery) is generally initiated only in patients with established disease and a well developed pathology. This "wait and see" approach precludes early intervention, and allows the progression of disease in the 25% who follow a complicated course. Therefore, within one embodiment of the invention, topical administration of anti-angiogenic factors (or anti-angiogenic
10 compositions, as described above) may be accomplished in infants which are at high risk for developing this condition in an attempt to cut down on the incidence of progression of retrolental fibroplasia. Within other embodiments, intravitreal injections and/or intraocular implants of an anti-angiogenic composition may be utilized. Such methods are particularly preferred in cases
15 of established disease, in order to reduce the need for surgery.

Within another aspect of the present invention, methods are provided for treating rheumatoid arthritis, comprising the step of administering a therapeutically effective amount of an anti-angiogenic composition to a joint, such that the formation of blood vessels is inhibited.

20 Briefly, in rheumatoid arthritis articular damage is due to a combination of inflammation (including white blood cells and white blood cell products) and pannus tissue development (a tissue composed of neovascular tissue, connective tissue, and inflammatory cells). Generally, chronic inflammation in itself is insufficient to result in damage to the joint surface, but
25 a permanent deficit is created once fibrovascular tissue digests the cartilage tissue.

Within a preferred embodiment of the invention, anti-angiogenic factors (including anti-angiogenic compositions, as described above) may be administered by intra-articular injection, as a surgical paste, or as an oral agent
30 (*e.g.*, containing the anti-angiogenic factor thalidomide), in order to inhibit the formation of blood vessels within the joint. One representative example of such a method is set forth in more detail below in Example 19.

As noted above, within yet another aspect of the present invention, vascular grafts are provided comprising a synthetic tube, the surface
35 of which is coated with an anti-angiogenic composition as described above. Briefly, vascular grafts are synthetic tubes, usually made of Dacron or Gortex, inserted surgically to bypass arterial blockages, most frequently from the aorta

to the femoral, or the femoral to the popliteal artery. A major problem which particularly complicates femoral-popliteal bypass grafts is the formation of a subendothelial scar-like reaction in the blood vessel wall called neointimal hyperplasia, which narrows the lumen within and adjacent to either end of the graft, and which can be progressive. A graft coated with or containing anti-angiogenic factors (or anti-angiogenic compositions, as described above) may be utilized to limit the formation of neointimal hyperplasia at either end of the graft. The graft may then be surgically placed by conventional bypass techniques.

Anti-angiogenic compositions of the present invention may also be utilized in a variety of other manners. For example, they may be incorporated into surgical sutures in order to prevent stitch granulomas, implanted in the uterus (in the same manner as an IUD) for the treatment of menorrhagia or as a form of female birth control, administered as either a peritoneal lavage fluid or for peritoneal implantation in the treatment of endometriosis, attached to a monoclonal antibody directed against activated endothelial cells as a form of systemic chemotherapy, or utilized in diagnostic imaging when attached to a radioactively labelled monoclonal antibody which recognizes activated endothelial cells.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF ANTI-INVASIVE FACTOR

5

The shoulder girdle and skull from a dogfish is excised, then scraped with a scalpel in order to remove all muscle and associated connective tissue from the cartilage. The cartilage is then homogenized with a tissue grinder, and extracted by continuous stirring at room temperature for 2 to 5 days in a solution containing 2.0 M guanidium hydrochloride and 0.02 M MES at pH 6.0.

After 2 to 5 days, the cartilage extract is passed through gauze netting in order to remove the larger constituents. The filtrate is then passed through an Amicon ultrafiltration unit which utilizes spiral-wound cartridges, with a molecular weight cutoff of 100,000. The filtrate (containing proteins with a molecular weight of less than 100,000 daltons) is then dialyzed against 0.02 M MES buffer (pH 6) with an Amicon ultrafiltration unit which retains proteins with a molecular weight of greater than 3,000 daltons. Utilizing this method, low molecular weight proteins and constituents are removed, as well as excessive amounts of guanidium HCl. The dialysate is concentrated to a final concentration 9 mg/ml.

EXAMPLE 2

25

ANALYSIS OF VARIOUS AGENTS FOR ANTI-ANGIOGENIC ACTIVITY

A. Chick Chorioallantoic Membrane ("Cam") Assays

Fertilized, domestic chick embryos were incubated for 3 days prior to shell-less culturing. In this procedure, the egg contents were emptied by removing the shell located around the air space. The interior shell membrane was then severed and the opposite end of the shell was perforated to allow the contents of the egg to gently slide out from the blunted end. The egg contents were emptied into round-bottom sterilized glass bowls and covered with petri dish covers. These were then placed into an incubator at 90% relative humidity and 3% CO₂ and incubated for 3 days.

Taxol (Sigma, St. Louis, MI) was mixed at concentrations of 1, 5, 10, 30mg per 10ml aliquot of 0.5% aqueous methylcellulose. Since taxol is insoluble in water, glass beads were used to produce fine particles. Ten microliter aliquots of this solution were dried on parafilm for 1 hour forming
5 disks 2mm in diameter. The dried disks containing taxol were then carefully placed at the growing edge of each CAM at day 6 of incubation. Controls were obtained by placing taxol-free methylcellulose disks on the CAMs over the same time course. After a 2 day exposure (day 8 of incubation) the vasculature was examined with the aid of a stereomicroscope. Liposyn II, a white opaque
10 solution, was injected into the CAM to increase the visibility of the vascular details. The vasculature of unstained, living embryos were imaged using a Zeiss stereomicroscope which was interfaced with a video camera (Dage-MTI Inc., Michigan City, IN). These video signals were then displayed at 160 times magnification and captured using an image analysis system (Vidas, Kontron;
15 Etching, Germany). Image negatives were then made on a graphics recorder (Model 3000; Matrix Instruments, Orangeburg, NY).

The membranes of the 8 day-old shell-less embryo were flooded with 2% glutaraldehyde in 0.1M Na cacodylate buffer; additional fixative was injected under the CAM. After 10 minutes *in situ*, the CAM was removed and
20 placed into fresh fixative for 2 hours at room temperature. The tissue was then washed overnight in cacodylate buffer containing 6% sucrose. The areas of interest were postfixied in 1% osmium tetroxide for 1.5 hours at 4°C. The tissues were then dehydrated in a graded series of ethanols, solvent exchanged with propylene oxide, and embedded in Spurr resin. Thin sections were cut with
25 a diamond knife, placed on copper grids, stained, and examined in a Joel 1200EX electron microscope. Similarly, 0.5 mm sections were cut and stained with toluidine blue for light microscopy.

At day 11 of development, chick embryos were used for the corrosion casting technique. Mercor resin (Ted Pella, Inc., Redding, CA) was
30 injected into the CAM vasculature using a 30-gauge hypodermic needle. The casting material consisted of 2.5 grams of Mercor CL-2B polymer and 0.05 grams of catalyst (55% benzoyl peroxide) having a 5 minute polymerization time. After injection, the plastic was allowed to sit *in situ* for an hour at room temperature and then overnight in an oven at 65°C. The CAM was then placed
35 in 50% aqueous solution of sodium hydroxide to digest all organic components. The plastic casts were washed extensively in distilled water, air-dried, coated

with gold/palladium, and viewed with the Philips 501B scanning electron microscope.

Results of the above experiments are shown in Figures 1-4. Briefly, the general features of the normal chick shell-less egg culture are shown in Figure 1A. At day 6 of incubation, the embryo is centrally positioned to a radially expanding network of blood vessels; the CAM develops adjacent to the embryo. These growing vessels lie close to the surface and are readily visible making this system an idealized model for the study of angiogenesis. Living, unstained capillary networks of the CAM can be imaged noninvasively with a stereomicroscope. Figure 1B illustrates such a vascular area in which the cellular blood elements within capillaries were recorded with the use of a video/computer interface. The 3-dimensional architecture of such CAM capillary networks is shown by the corrosion casting method and viewed in the scanning electron microscope (Figure 1C). These castings revealed underlying vessels which project toward the CAM surface where they form a single layer of anastomotic capillaries.

Transverse sections through the CAM show an outer ectoderm consisting of a double cell layer, a broader mesodermal layer containing capillaries which lie subjacent to the ectoderm, adventitial cells, and an inner, single endodermal cell layer (Figure 1D). At the electron microscopic level, the typical structural details of the CAM capillaries are demonstrated. Typically, these vessels lie in close association with the inner cell layer of ectoderm (Figure 1E)

After 48 hours exposure to taxol at concentrations of 1, 5, 10, or 30 mg, each CAM was examined under living conditions with a stereomicroscope equipped with a video/computer interface in order to evaluate the effects on angiogenesis. This imaging setup was used at a magnification of 160 times which permitted the direct visualization of blood cells within the capillaries; thereby blood flow in areas of interest could be easily assessed and recorded. For this study, the inhibition of angiogenesis was defined as an area of the CAM devoid of a capillary network ranging from 2 - 6 mm in diameter. Areas of inhibition lacked vascular blood flow and thus were only observed under experimental conditions of methylcellulose containing taxol; under control conditions of disks lacking taxol there was no effect on the developing capillary system. The dose-dependent, experimental data of the effects of taxol at different concentrations are shown in Table II.

TABLE II

Angiogenic Inhibition by Taxol		
Taxol Concentration μg	Embryos Evaluated (Positive/Total)	% Inhibition
30	31/31	100
10	16/21	76
5	18/25	72
1	6/15	40
Control	0/30	0

Typical taxol-treated CAMs (Figures 2A and 2B) are shown with the transparent methylcellulose disk centrally positioned over the avascular zone measuring 6 mm in diameter. At a slightly higher magnification, the periphery of such avascular zones is clearly evident (Figure 2C); the surrounding functional vessels were often redirected away from the source of taxol (Figures 2C and 2D). Such angular redirecting of blood flow was never observed under normal conditions. Another feature of the effects of taxol was the formation of blood islands within the avascular zone representing the aggregation of blood cells.

The associated morphological alterations of the taxol-treated CAM are readily apparent at both the light and electron microscopic levels. For the convenience of presentation, three distinct phases of general transition from the normal to the avascular state are shown. Near the periphery of the avascular zone the CAM is hallmarked by an abundance of mitotic cells within all three germ layers (Figures 3A and 4A). This enhanced mitotic division was also a consistent observation for capillary endothelial cells. However, the endothelial cells remained junctionally intact with no extravasation of blood cells. With further degradation, the CAM is characterized by the breakdown and dissolution of capillaries (Figures 3B and 4B). The presumptive endothelial cells, typically arrested in mitosis, still maintain a close spatial relationship with blood cells and lie subjacent to the ectoderm; however, these cells are not junctionally linked. The most central portion of the avascular zone was characterized by a thickened ectodermal and endodermal layer (Figures 3C and 4C). Although these layers were thickened, the cellular junctions remained

intact and the layers maintained their structural characteristics. Within the mesoderm, scattered mitotically arrested cells were abundant; these cells did not exhibit the endothelial cell polarization observed in the former phase. Also, throughout this avascular region, degenerating cells were common as noted by
5 the electron dense vacuoles and cellular debris (Figure 4C).

In summary, this study demonstrated that 48 hours after taxol application to the CAM, angiogenesis was inhibited. The blood vessel inhibition formed an avascular zone which was represented by three transitional phases of
10 taxol's effect. The central, most affected area of the avascular zone contained disrupted capillaries with extravasated red blood cells; this indicated that intercellular junctions between endothelial cells were absent. The cells of the endoderm and ectoderm maintained their intercellular junctions and therefore these germ layers remained intact; however, they were slightly thickened. As
15 the normal vascular area was approached, the blood vessels retained their junctional complexes and therefore also remained intact. At the periphery of the taxol-treated zone, further blood vessel growth was inhibited which was evident by the typical redirecting or "elbowing" effect of the blood vessels (Figure 24D).

20 Taxol-treated avascular zones also revealed an abundance of cells arrested in mitosis in all three germ layers of the CAM; this was unique to taxol since no previous study has illustrated such an event. By being arrested in mitosis, endothelial cells could not undergo their normal metabolic functions involved in angiogenesis. In comparison, the avascular zone formed by suramin
25 and cortisone acetate do not produce mitotically arrested cells in the CAM; they only prevented further blood vessel growth into the treated area. Therefore, even though agents are anti-angiogenic, there are many points in which the angiogenesis process may be targeted.

We also observed the effects of taxol over the 48 hour duration
30 and noticed that inhibition of angiogenesis occurs as early as 9 hours after application. Histological sections revealed a similar morphology as seen in the first transition phase of the avascular zone at 48 hours illustrated in figure 3a and 4a. Also, we observed the revascularization process into the avascular zone previously observed. It has been found that the avascular zone formed by
35 heparin and angiostatic steroids became revascularized 60 hours after application. In our study, taxol-treated avascular zones did not revascularize for at least 7 days after application implying a more potent long-term effect.

EXAMPLE 3

ENCAPSULATION OF SURAMIN

5

One milliliter of 5% ELVAX (poly(ethylene-vinyl acetate) cross-linked with 5% vinyl acetate) in dichloromethane ("DCM") is mixed with a fixed weight of sub-micron ground sodium suramin. This mixture is injected into 5 ml of 5% Polyvinyl Alcohol ("PVA") in water in a 30 ml flat bottomed test tube. Tubes containing different weights of the drug are then suspended in a multi-sample water bath at 40° for 90 minutes with automated stirring. The mixes are removed, and microsphere samples taken for size analysis. Tubes are centrifuged at 1000g for 5 min. The PVA supernatant is removed and saved for analysis (nonencapsulated drug). The microspheres are then washed (vortexed) in 5 ml of water and recentrifuged. The 5 ml wash is saved for analysis (surface bound drug). Microspheres are then wetted in 50 ul of methanol, and vortexed in 1 ml of DCM to dissolve the ELVAX. The microspheres are then warmed to 40°C, and 5 ml of 50°C water is slowly added with stirring. This procedure results in the immediate evaporation of DCM, thereby causing the release of sodium suramin into the 5 ml of water. All three 5 ml samples were then assayed for drug content.

Sodium suramin absorbs uv/vis with a lambda max of 312nm. The absorption is linear in the 0 to 100 ug/ml range in both water and 5% PVA. The drug fluoresces strongly with an excitation maximum at 312nm, and emission maximum at 400nm. This fluorescence is quantifiable in the 0 to 25 ug/ml range.

Results are shown in Figures 5-10. Briefly, the size distribution of microspheres appears to be unaffected by inclusion of the drug in the DCM (see Figures 5 and 6). Good yields of microspheres in the 20 to 60 μ m range may be obtained.

The encapsulation of suramin is very low (<1%) (see Figure 8). However as the weight of drug is increased in the DCM the total amount of drug encapsulated increased although the % encapsulation decreased. As is shown in Figure 7, 50ug of drug may be encapsulated in 50 mg of ELVAX. Encapsulation of sodium suramin in 5% PVA containing 10% NaCl is shown in Figures 9-10.

EXAMPLE 4

ENCAPSULATION OF TAXOL

5 Five hundred micrograms of either taxol or baccatin (a taxol analog, available from Inflazyme Pharmaceuticals Inc., Vancouver, British Columbia, Canada) are dissolved in 1 ml of a 50:50 ELVAX:poly-l-lactic acid mixture in dcm. Microspheres are then prepared in a dissolution machine (Six-spindle dissolution tester, VanderKamp, Van Kell Industries Inc., U.S.A.) in
10 triplicate at 200 rpm, 42°C, for 3 hours. Microspheres so prepared are washed twice in water and sized on the microscope.

Determination of taxol encapsulation is undertaken in a uv/vis assay (uv/vis lamda max. at 237 nm, fluorescence assay at excitation 237, emission at 325 nm; Fluorescence results are presented in square brackets []).
15 Utilizing the procedures described above, 58 μg (+/-12 μg) [75 μg (+/-25 μg)] of taxol may be encapsulated from a total 500 μg of starting material. This represents 12% (+/-2.4%) [15% (+/-5%)] of the original weight, or 1.2% (+/-0.25%) [1.5% (+/-0.5%)] by weight of the polymer. After 18 hours of tumbling in an oven at 37°C, 10.3% (+/-10%) [6% (+/-5.6%)] of the total taxol had been
20 released from the microspheres.

For baccatin, 100 +/-15 μg [83 +/-23 μg] of baccatin can be encapsulated from a total of 500 μg starting material. This represents a 20% (+/-3%) [17% (+/-5%)] of the original weight of baccatin, and 2% (+/-0.3%) [1.7% (+/-0.5%)] by weight of the polymer. After 18 hours of tumbling in an
25 oven at 37°C, 55% (+/-13%) [60% (+/- 23%)] of the baccatin is released from the microspheres.

EXAMPLE 5ANALYSIS OF SURGICAL PASTE CONTAINING
ANTI-ANGIOGENIC COMPOSITIONS

30 Fisher rats weighing approximately 300 grams are anesthetized, and a 1 cm transverse upper abdominal incision is made. Two-tenths of a milliliter of saline containing 1×10^6 live 9L gliosarcoma cells (eluted immediately prior to use from tissue culture) are injected into 2 of the 5 hepatic
35 lobes by piercing a 27 gauge needle 1 cm through the liver capsule. The

abdominal wound is closed with 6.0 resorbable suture and skin clips and the GA terminated.

After 2 weeks, the tumor deposits will measure approximately 1 cm. At this time, both hepatic tumors are resected and the bare margin of the liver is packed with a hemostatic agent. The rats are divided into two groups: half is administered polymeric carrier alone, and the other half receives an anti-angiogenic composition.

Rats are sacrificed 2, 7, 14, 21 and 84 days post hepatic resection. In particular, the rats are euthanized by injecting Euthanyl into the dorsal vein of the tail. The liver, spleen, and both lungs are removed, and histologic analysis is performed in order to study the tumors for evidence of anti-angiogenic activity.

15

EXAMPLE 6

EMBOLIZATION OF RAT ARTERIES

Fisher rats weighing approximately 300 grams are anesthetized. Utilizing aseptic procedures, a 1 cm transverse upper abdominal incision is made, and the liver identified. Two-tenths of a milliliter of saline containing 1 million live 9L gliosarcoma cells (eluted immediately prior from tissue culture) is injected into each of the 5 hepatic lobes by piercing a 27 gauge needle 1 cm through the liver capsule. One-tenth of a milliliter of normal saline is injected into the needle as it is withdrawn to ensure that there is no spillage of cells into the peritoneal cavity. A pledget of gelfoam is placed on each of the puncture sites to ensure hemostasis. The abdominal wound is closed with 6.0 resorbable suture with skin clips, and the anesthetic terminated. The rat is returned to the animal care facility to have a standard diet for 14 days, at which time each tumor deposit will measure 1 cm in diameter. The same procedure is repeated using Westar rats and a Colon Cancer cell line (Radiologic Oncology Lab, M.D. Anderson, Houston, Texas). In this instance, 3 weeks are required post-injection for the tumor deposits to measure 1 cm in diameter each.

After 2 or 3 weeks, depending on the rat species, the same general anesthetic procedure is followed and a midline abdominal incision is performed. The duodenum is flipped and the gastroduodenal artery is identified and mobilized. Ties are placed above and below a cutdown site on the midportion of the gastroduodenal artery (GDA), and 0.038 inch polyethylene tubing is

introduced in a retrograde fashion into the artery using an operating microscope. The tie below the insertion point will ligate the artery, while the one above will fix the catheter in place. Angiography is performed by injecting 0.5 ml of 60% radiopaque contrast material through the catheter as an x-ray is taken. The hepatic artery is then embolized by refluxing particles measuring 15-200 μm through the gastroduodenal artery catheter until flow, observed via the operating microscope, is seen to cease for at least 30 seconds. Occlusion of the hepatic artery is confirmed by repeating an angiogram through the GDA catheter. Utilizing this procedure, one-half of the rats receive 15-200 μm particles of polymer alone, and the other half receive 15-200 μm particles of the polymer-anti-angiogenic factor composition. The upper GDA ligature is tightened to occlude the GDA as the catheter is withdrawn to ensure hemostasis, and the hepatic artery (although embolized) is left intact. The abdomen is closed with 6.0 absorbable suture and surgical clips.

The rats are subsequently sacrificed at 2, 7, 14, 21 and 84 days post-embolization in order to determine efficacy of the anti-angiogenic factor. Briefly, general anesthetic is given, and utilizing aseptic precautions, a midline incision performed. The GDA is mobilized again, and after placing a ligature near the junction of the GDA and the hepatic artery (*i.e.*, well above the site of the previous cutdown), a 0.038-inch polyethylene tubing is inserted via cutdown of the vessel and angiography is performed. The rat is then euthanized by injecting Euthanyl into the dorsal vein of the tail. Once euthanasia is confirmed, the liver is removed *en bloc* along with the stomach, spleen and both lungs.

Histologic analysis is performed on a prepared slide stained with hematoxylin and eosin ("H and E") stain. Briefly, the lungs are sectioned at 1 cm intervals to assess passage of embolic material through the hepatic veins and into the right side of circulation. The stomach and spleen are also sectioned in order to assess inadvertent immobilization from reflux of particles into the celiac access of the collateral circulation.

EXAMPLE 7

TRANSPLANTATION OF BILIARY STENTS IN RATS

General anesthetic is administered to 300 gram Fisher rats. A 1 cm transverse incision is then made in the upper abdomen, and the liver identified. In the most superficial lobe, 0.2 ml of saline containing 1 million

cells of 9L gliosarcoma cells (eluted from tissue culture immediately prior to use) is injected via a 27 gauge needle to a depth of 1 cm into the liver capsule. Hemostasis is achieved after removal of the needle by placing a pledget of gelfoam at the puncture sites. Saline is injected as the needle is removed to ensure no spillage of cells into the peritoneal cavity or along the needle track. The general anesthetic is terminated, and the animal returned to the animal care center and placed on a normal diet.

Two weeks later, general anesthetic is administered, and utilizing aseptic precautions, the hepatic lobe containing the tumor is identified through a midline incision. A 16 gauge angiographic needle is then inserted through the hepatic capsule into the tumor, a 0.038-inch guidewire passed through the needle, and the needle withdrawn over the guidewire. A number 5 French dilator is passed over the guide into the tumor and withdrawn. A number 5 French delivery catheter is then passed over the wire containing a self-expanding stainless steel Wallstent (5 mm in diameter and 1 cm long). The stent is deployed into the tumor and the guidewire delivery catheter is removed. One-third of the rats have a conventional stainless steel stent inserted into the tumor, one-third a stainless steel stent coated with polymer, and one third a stent coated with the polymer-anti-angiogenic factor compound. The general anesthetic is terminated and the rat returned to the animal care facility.

A plain abdominal X-ray is performed at 2 days in order to assess the degree of stent opening. Rats are sacrificed at 2, 7, 14, 28 and 56 days post-stent insertion by injecting Euthanyl, and their livers removed *en bloc* once euthanasia is confirmed. After fixation in formaldehyde for 48 hours, the liver is sectioned at 0.5 mm intervals; including severing the stent transversely using a fresh blade for each slice. Histologic sections stained with H and E are then analyzed to assess the degree of tumor ingrowth into the stent lumen.

30

EXAMPLE 8

MANUFACTURE OF MICROSPHERES

Equipment which is preferred for the manufacture of microspheres described below include: 200 ml water jacketed beaker (Kimax or Pyrex), Haake circulating water bath, overhead stirrer and controller with 2 inch diameter (4 blade, propeller type stainless steel stirrer - Fisher brand), 500 ml glass beaker, hot plate/stirrer (Corning brand), 4 X 50 ml polypropylene

centrifuge tubes (Nalgene), glass scintillation vials with plastic insert caps, table top centrifuge (GPR Beckman), high speed centrifuge- floor model (JS 21 Beckman), Mettler analytical balance (AJ 100, 0.1 mg), Mettler digital top loading balance (AE 163, 0.01 mg), automatic pipetter (Gilson). Reagents include Polycaprolactone ("PCL" - mol wt 10,000 to 20,000; Polysciences, Warrington Pennsylvania, USA), "washed" Ethylene Vinyl Acetate ("EVA" washed so as to remove the anti-oxidant BHT), Poly(DL)lactic acid ("PLA" - mol wt 15,000 to 25,000; Polysciences), Polyvinyl Alcohol ("PVA" - mol wt 124,000 to 186,000; 99% hydrolyzed; Aldrich Chemical Co., Milwaukee WI, USA), Dichloromethane ("DCM" or "methylene chloride"; HPLC grade Fisher scientific), and distilled water.

A. Preparation of 5% (w/v) Polymer Solutions

Depending on the polymer solution being prepared, 1.00 g of PCL or PLA, or 0.50 g each of PLA and washed EVA is weighed directly into a 20 ml glass scintillation vial. Twenty milliliters of DCM is then added, and the vial tightly capped. The vial is stored at room temperature (25°C) for one hour (occasional shaking may be used), or until all the polymer has dissolved (the solution should be clear). The solution may be stored at room temperature for at least two weeks.

B. Preparation of 5% (w/v) Stock Solution of PVA

Twenty-five grams of PVA is weighed directly into a 600 ml glass beaker. Five hundred milliliters of distilled water is added, along with a 3 inch Teflon coated stir bar. The beaker is covered with glass to decrease evaporation losses, and placed into a 2000 ml glass beaker containing 300 ml of water (which acts as a water bath). The PVA is stirred at 300 rpm at 85°C (Corning hot plate/stirrer) for 2 hours or until fully dissolved. Dissolution of the PVA may be determined by a visual check; the solution should be clear. The solution is then transferred to a glass screw top storage container and stored at 4°C for a maximum of two months. The solution, however should be warmed to room temperature before use or dilution.

C. Procedure for Producing Microspheres

Based on the size of microspheres being made (see Table 1), 100 ml of the PVA solution (concentrations given in Table III) is placed into the 200 ml water jacketed beaker. Haake circulating water bath is connected to this beaker and the contents are allowed to equilibrate at 27°C (+/-10°C) for 10 minutes. Based on the size of microspheres being made (see Table III), the start speed of the overhead stirrer is set, and the blade of the overhead stirrer placed half way down in the PVA solution. The stirrer is then started, and 10 ml of polymer solution (polymer solution used based on type of microspheres being produced) is then dripped into the stirring PVA over a period of 2 minutes using a 5 ml automatic pipetter. After 3 minutes the stir speed is adjusted (see Table III), and the solution stirred for an additional 2.5 hours. The stirring blade is then removed from the microsphere preparation, and rinsed with 10 ml of distilled water so that the rinse solution drains into the microsphere preparation. The microsphere preparation is then poured into a 500 ml beaker, and the jacketed water bath washed with 70 ml of distilled water, which is also allowed to drain into the microsphere preparation. The 180 ml microsphere preparation is then stirred with a glass rod, and equal amounts are poured into four polypropylene 50 ml centrifuge tubes. The tubes are then capped, and centrifuged for 10 minutes (force given in Table 1). A 5 ml automatic pipetter or vacuum suction is then utilized to draw 45 ml of the PVA solution off of each microsphere pellet.

TABLE III

PVA concentrations, stir speeds, and centrifugal force requirements for each diameter range of microspheres.

5 10 15 20 25	PRODUCTION STAGE	MICROSPHERE DIAMETER RANGES		
		30 μm to 100 μm	10 μm to 30 μm	0.1 μm to 3 μm
	PVA Concentration	2.5% (w/v) (i.e., dilute 5% stock with distilled water)	5% (w/v) (i.e., undiluted stock)	3.5% (w/v) (i.e., dilute 5% stock with distilled water)
	Starting Stir Speed	500 rpm +/- 50 rpm	500 rpm +/- 50 rpm	3000 rpm +/- 200 rpm
	Adjusted Stir Speed	500 rpm +/- 50 rpm	500 rpm +/- 50 rpm	2500 rpm +/- 200 rpm
	Centrifuge Force	1000 g +/- 100 g (Table top model)	1000 g +/- 100 g (Table top model)	10 000 g +/- 1000 g (High speed model)

Five milliliters of distilled water is then added to each centrifuge tube, which is then vortexed to resuspend the microspheres. The four microsphere suspensions are then pooled into one centrifuge tube along with 20 ml of distilled water, and centrifuged for another 10 minutes (force given in Table 1). This process is repeated two additional times for a total of three washes. The microspheres are then centrifuged a final time, and resuspended in 10 ml of distilled water. After the final wash, the microsphere preparation is transferred into a preweighed glass scintillation vial. The vial is capped, and left overnight at room temperature (25°C) in order to allow the microspheres to sediment out under gravity. Microspheres which fall in the size range of 0.1 μm to 3 μm do not sediment out under gravity, so they are left in the 10 ml suspension.

D. Drying of 10 μm to 30 μm or 30 μm to 100 μm Diameter Microspheres

After the microspheres have sat at room temperature overnight, a
5 5 ml automatic pipetter or vacuum suction is used to draw the supernatant off of
the sedimented microspheres. The microspheres are allowed to dry in the
uncapped vial in a drawer for a period of one week or until they are fully dry
(vial at constant weight). Faster drying may be accomplished by leaving the
uncapped vial under a slow stream of nitrogen gas (flow approx. 10 ml/min.) in
10 the fume hood. When fully dry (vial at constant weight), the vial is weighed and
capped. The labelled, capped vial is stored at room temperature in a drawer.
Microspheres are normally stored no longer than 3 months.

E. Drying of 0.1 μm to 3 μm Diameter Microspheres

15

This size range of microspheres will not sediment out, so they are
left in suspension at 4°C for a maximum of four weeks. To determine the
concentration of microspheres in the 10 ml suspension, a 200 μl sample of the
suspension is pipetted into a 1.5 ml preweighed microfuge tube. The tube is
20 then centrifuged at 10,000 g (Eppendorf table top microfuge), the supernatant
removed, and the tube allowed to dry at 50°C overnight. The tube is then
reweighed in order to determine the weight of dried microspheres within the
tube.

25

F. Manufacture of Taxol Loaded Microsphere

In order to prepare taxol containing microspheres, an appropriate
amount of weighed taxol (based upon the percentage of taxol to be
encapsulated) is placed directly into a 20 ml glass scintillation vial. Ten
30 milliliters of an appropriate polymer solution is then added to the vial
containing the taxol, which is then vortexed until the taxol has dissolved.

Microspheres containing taxol may then be produced essentially
as described above in steps (C) through (E).

35

EXAMPLE 9

MANUFACTURE OF STENT COATING

Reagents and equipment which are utilized within the following experiments include (medical grade stents obtained commercially from a variety of manufacturers; e.g., the "Strecker" stent) and holding apparatus, 20 ml glass scintillation vial with cap (plastic insert type), TLC atomizer, Nitrogen gas tank, glass test tubes (various sizes from 1 ml and up), glass beakers (various sizes), Pasteur pipette, tweezers, Polycaprolactone ("PCL" - mol wt 10,000 to 20,000; Polysciences), Taxol (Sigma Chemical Co., St. Louis, Mo., 95% purity), Ethylene vinyl acetate ("EVA" - washed - see previous), Poly(DL)lactic acid ("PLA" - mol wt 15,000 to 25,000; Polysciences), dichloromethane ("DCM" - HPLC grade, Fisher Scientific).

15 A. Procedure for Sprayed Stents

The following describes a typical method using a 3 mm crimped diameter interleaving metal wire stent of approximately 3 cm length. For larger diameter stents, larger volumes of polymer/drug solution are used.

20 Weigh sufficient polymer directly into a 20 ml glass scintillation vial and add sufficient DCM to achieve a 2% w/v solution. Cap the vial and mix the solution to dissolve the polymer (hand shaking). Assemble the stent in a vertical orientation. This can be accomplished using a piece of nylon and tying the stent to a retort stand. Position this stent holding apparatus 6 to 12 inches
25 above the fume hood floor on a suitable support (e.g., inverted 2000 ml glass beaker) to enable horizontal spraying. Using an automatic pipette, transfer a suitable volume (minimum 5 ml) of the 2% polymer solution to a separate 20 ml glass scintillation vial. Add an appropriate amount of taxol to the solution and dissolve it by hand shaking the capped vial.

30 To prepare for spraying, remove the cap of this vial and dip the barrel (only) of an TLC atomizer into the polymer solution. Note that the reservoir of the atomizer need not be used in this procedure: the 20 ml glass vial acts as a reservoir. Connect the nitrogen tank to the gas inlet of the atomizer. Gradually increase the pressure until atomization and spraying
35 begins. Note the pressure and use this pressure throughout the procedure. To spray the stent use 5 second oscillating sprays with a 15 second dry time between sprays. After 5 sprays, rotate the stent 90° and spray that portion of the stent.

Repeat until all sides of the stent have been sprayed. During the dry time, finger crimp the gas line to avoid wastage of the spray. Spraying is continued until a suitable amount of polymer is deposited on the stents. The amount may be based on the specific stent application *in vivo*. To determine the amount, weigh the stent after spraying has been completed and the stent has dried. Subtract the original weight of the stent from the finished weight and this produces the amount of polymer (plus taxol) applied to the stent. Store the coated stent in a sealed container.

10 B. Procedure for Dipped Stents

The following describes a typical method using a 3 mm crimped diameter interleaving metal wire stent of approximately 3 cm length. For larger diameter stents, larger volumes of polymer/drug solution are used in larger sized test tubes.

15 Weigh 2 g of EVA into a 20 ml glass scintillation vial and add 20 ml of DCM. Cap the vial and leave it for 2 hours to dissolve (hand shake the vial frequently to assist the dissolving process). Weigh a known weight of taxol directly into a 1 ml glass test tube and add 0.5 ml of the polymer solution. Using a glass Pasteur pipette, dissolve the taxol by gently pumping the polymer solution. Once the taxol is dissolved, hold the test tube in a near horizontal position (the sticky polymer solution will not flow out). Using tweezers, insert the stent into the tube all the way to the bottom. Allow the polymer solution to flow almost to the mouth of the test tube by angling the mouth below horizontal and then restoring the test tube to an angle slightly above the horizontal. While slowly rotating the stent in the tube, slowly remove the stent (approximately 30 seconds).

Hold the stent in a vertical position to dry. Some of the *sealed* perforations may *pop* so that a hole exists in the continuous sheet of polymer. This may be remedied by repeating the previous dipping procedure, however repetition of the procedure can also lead to further popping and a general uneven build up of polymer. Generally, it is better to dip the stent just once and to cut out a section of stent that has no *popped* perforations. Store the dipped stent in a sealed container.

35

EXAMPLE 10

MANUFACTURE OF SURGICAL "PASTES"

As noted above, the present invention provides a variety of
5 polymeric-containing drug compositions that may be utilized within a variety of
clinical situations. For example, compositions may be produced: (1) as a
"thermopaste" that is applied to a desired site as a fluid, and hardens to a solid
of the desired shape at a specified temperature (*e.g.*, body temperature); (2) as a
10 spray (*i.e.*, "nanospray") which may delivered to a desired site either directly or
through a specialized apparatus (*e.g.*, endoscopy), and which subsequently
hardens to a solid which adheres to the tissue to which it is applied; (3) as an
adherent, pliable, resilient, angiogenesis inhibitor-polymer film applied to a
desired site either directly or through a specialized apparatus, and which
preferably adheres to the site to which it is applied; and (4) as a fluid composed
15 of a suspension of microspheres in an appropriate carrier medium, which is
applied to a desired site either directly or via a specialized apparatus, and which
leaves a layer of microspheres at the application site. Representative examples
of each of the above embodiments is set forth in more detail below.

20 A. Procedure for Producing Thermopaste

Reagents and equipment which are utilized within the following
experiments include a sterile glass syringe (1 ml), Corning hot plate/stirrer, 20
ml glass scintillation vial, moulds (*e.g.*, 50 μ l DSC pan or 50 ml centrifuge tube
25 cap inner portion), scalpel and tweezers, Polycaprolactone ("PCL" - mol wt
10,000 to 20,000; Polysciences, Warrington, Pennsylvania USA), and Taxol
(Sigma grade 95% purity minimum).

Weigh 5.00 g of polycaprolactone directly into a 20 ml glass
scintillation vial. Place the vial in a 600 ml beaker containing 50 ml of water.
30 Gently heat the beaker to 65°C and hold it at that temperature for 20 minutes.
This allows the polymer to melt. Thoroughly mix a known weight of taxol, or
other angiogenesis inhibitor into the melted polymer at 65°C. Pour the melted
polymer into a prewarmed (60°C oven) mould. Use a spatula to assist with the
pouring process. Allow the mould to cool so the polymer solidifies. Cut or
35 break the polymer into small pieces (approximately 2 mm by 2 mm in size).
These pieces must fit into a 1 ml glass syringe. Remove the plunger from the 1

ml glass syringe (do not remove the cap from the tip) and place it on a balance. Zero the balance.

Weigh 0.5 g of the pieces directly into the open end of the syringe. Place the glass syringe upright (capped tip downwards) into a 500 ml glass beaker containing distilled water at 65°C (corning hot plate) so that no water enters the barrel. The polymer melts completely within 10 minutes in this apparatus. When the polymer pieces have melted, remove the barrel from the water bath, hold it horizontally and remove the cap. Insert the plunger into the barrel and compress the melted polymer into a sticky mass at the tip end of the barrel. Cap the syringe and allow it to cool to room temperature.

For application, the syringe may be reheated to 60°C and administered as a liquid which solidifies when cooled to body temperature.

B. Procedure for Producing Nanospray

Nanospray is a suspension of small microspheres in saline. If the microspheres are very small (*i.e.*, under 1 μm in diameter) they form a colloid so that the suspension will not sediment under gravity. As is described in more detail below, a suspension of 0.1 μm to 1 μm microparticles may be created suitable for deposition onto tissue through a finger pumped aerosol. Equipment and materials which may be utilized to produce nanospray include 200 ml water jacketed beaker (Kimax or Pyrex), Haake circulating water bath, overhead stirrer and controller with 2 inch diameter (4 blade, propeller type stainless steel stirrer; Fisher brand), 500 ml glass beaker, hot plate/stirrer (Corning brand), 4 X 50 ml polypropylene centrifuge tubes (Nalgene), glass scintillation vials with plastic insert caps, table top centrifuge (Beckman), high speed centrifuge - floor model (JS 21 Beckman), Mettler analytical balance (AJ 100, 0.1 mg), Mettler digital top loading balance (AE 163, 0.01 mg), automatic pipetter (Gilson), sterile pipette tips, pump action aerosol (Pfeiffer pharmaceuticals) 20 ml, laminar flow hood, Polycaprolactone ("PCL" - mol wt 10,000 to 20,000; Polysciences, Warrington, Pennsylvania USA), "washed" (see previous) Ethylene Vinyl Acetate ("EVA"), Poly(DL)lactic acid ("PLA" mol wt 15,000 to 25,000; Polysciences), Polyvinyl Alcohol ("PVA" - mol wt 124,000 to 186,000; 99% hydrolyzed; Aldrich Chemical Co., Milwaukee, WI USA), Dichloromethane ("DCM" or "methylene chloride;" HPLC grade Fisher scientific), Distilled water, sterile saline (Becton and Dickenson or equivalent)

1. *Preparation of 5% (w/v) Polymer Solutions*

Depending on the polymer solution being prepared, weigh 1.00 g of PCL or PLA or 0.50 g each of PLA and washed EVA directly into a 20 ml glass scintillation vial. Using a measuring cylinder, add 20 ml of DCM and tightly cap the vial. Leave the vial at room temperature (25°C) for one hour or until all the polymer has dissolved (occasional hand shaking may be used). Dissolving of the polymer can be determined by a visual check; the solution should be clear. Label the vial with the name of the solution and the date it was produced. Store the solutions at room temperature and use within two weeks.

2. *Preparation of 3.5% (w/v) Stock Solution of PVA*

The solution can be prepared by following the procedure given below, or by diluting the 5% (w/v) PVA stock solution prepared for production of microspheres (see Example 8). Briefly, 17.5 g of PVA is weighed directly into a 600 ml glass beaker, and 500 ml of distilled water is added. Place a 3 inch teflon coated stir bar in the beaker. Cover the beaker with a cover glass to reduce evaporation losses. Place the beaker in a 2000 ml glass beaker containing 300 ml of water. This will act as a water bath. Stir the PVA at 300 rpm at 85°C (Corning hot plate/stirrer) for 2 hours or until fully dissolved. Dissolving of the PVA can be determined by a visual check; the solution should be clear. Use a pipette to transfer the solution to a glass screw top storage container and store at 4°C for a maximum of two months. This solution should be warmed to room temperature before use or dilution.

3. *Procedure for Producing Nanospray*

Place the stirring assembly in a fume hood. Place 100 ml of the 3.5% PVA solution in the 200 ml water jacketed beaker. Connect the Haake water bath to this beaker and allow the contents to equilibrate at 27°C (+/-1°C) for 10 minutes. Set the start speed of the overhead stirrer at 3000 rpm (+/- 200 rpm). Place the blade of the overhead stirrer half way down in the PVA solution and start the stirrer. Drip 10 ml of polymer solution (polymer solution used based on type of nanospray being produced) into the stirring PVA over a period of 2 minutes using a 5 ml automatic pipetter. After 3 minutes, adjust the stir speed to 2500 rpm (+/- 200 rpm) and leave the assembly for 2.5 hours. After 2.5 hours, remove the stirring blade from the nanospray preparation and rinse with 10 ml of distilled water. Allow the rinse solution to go into the nanospray preparation.

Pour the microsphere preparation into a 500 ml beaker. Wash the jacketed water bath with 70 ml of distilled water. Allow the 70 ml rinse solution to go into the microsphere preparation. Stir the 180 ml microsphere preparation with a glass rod and pour equal amounts of it into four polypropylene 50 ml centrifuge tubes. Cap the tubes. Centrifuge the capped tubes at 10 000 g (+/- 1000 g) for 10 minutes. Using a 5 ml automatic pipetter or vacuum suction, draw 45 ml of the PVA solution off of each microsphere pellet and discard it. Add 5 ml of distilled water to each centrifuge tube and use a vortex to resuspend the microspheres in each tube. Using 20 ml of distilled water, pool the four microsphere suspensions into one centrifuge tube. To wash the microspheres, centrifuge the nanospray preparation for 10 minutes at 10 000 g (+/- 1000 g). Draw the supernatant off of the microsphere pellet. Add 40 ml of distilled water and use a vortex to resuspend the microspheres. Repeat this process two more times for a total of three washes. Do a fourth wash but use only 10 ml (not 40 ml) of distilled water when resuspending the microspheres. After the fourth wash, transfer the microsphere preparation into a preweighed glass scintillation vial.

Cap the vial and let it sit for 1 hour at room temperature (25°C) to allow the 2 μ m and 3 μ m diameter microspheres to sediment out under gravity. After 1 hour, draw off the top 9 ml of suspension using a 5 ml automatic pipetter. Place the 9 ml into a sterile capped 50 ml centrifuge tube. Centrifuge the suspension at 10 000 g (+/- 1000 g) for 10 minutes. Discard the supernatant and resuspend the pellet in 20 ml of sterile saline. Centrifuge the suspension at 10 000 g (+/- 1000 g) for 10 minutes. Discard the supernatant and resuspend the pellet in sterile saline. The quantity of saline used is dependent on the final required suspension concentration (usually 10% w/v). Thoroughly rinse the aerosol apparatus in sterile saline and add the nanospray suspension to the aerosol.

30 C. Manufacture of Taxol Loaded Nanospray

To manufacture nanospray containing taxol, use Taxol (Sigma grade 95% purity). To prepare the polymer drug stock solution, weigh the appropriate amount of taxol directly into a 20 ml glass scintillation vial. The appropriate amount is determined based on the percentage of taxol to be in the nanospray. For example, if nanospray containing 5% taxol was required, then

the amount of taxol weighed would be 25 mg since the amount of polymer added is 10 ml of a 5% polymer in DCM solution (see next step).

Add 10 ml of the appropriate 5% polymer solution to the vial containing the taxol. Cap the vial and vortex or hand swirl it to dissolve the taxol (visual check to ensure taxol dissolved). Label the vial with the date it was produced. This is to be used the day it is produced.

Follow the procedures as described above, except that polymer/drug (e.g., taxol) stock solution is substituted for the polymer solution.

10

D. Procedure for Producing Film

The term film refers to a polymer formed into one of many geometric shapes. The film may be a thin, elastic sheet of polymer or a 2 mm thick disc of polymer. This film is designed to be placed on exposed tissue so that any encapsulated drug is released from the polymer over a long period of time at the tissue site. Films may be made by several processes, including for example, by casting, and by spraying.

In the casting technique, polymer is either melted and poured into a shape or dissolved in dichloromethane and poured into a shape. The polymer then either solidifies as it cools or solidifies as the solvent evaporates, respectively. In the spraying technique, the polymer is dissolved in solvent and sprayed onto glass, as the solvent evaporates the polymer solidifies on the glass. Repeated spraying enables a build up of polymer into a film that can be peeled from the glass.

Reagents and equipment which were utilized within these experiments include a small beaker, Corning hot plate stirrer, casting moulds (e.g., 50 ml centrifuge tube caps) and mould holding apparatus, 20 ml glass scintillation vial with cap (Plastic insert type), TLC atomizer, Nitrogen gas tank, Polycaprolactone ("PCL" - mol wt 10,000 to 20,000; Polysciences), Taxol (Sigma 95% purity), Ethanol, "washed" (see previous) Ethylene vinyl acetate ("EVA"), Poly(DL)lactic acid ("PLA" - mol wt 15,000 to 25,000; Polysciences), Dichloromethane (HPLC grade Fisher Scientific).

1. *Procedure for Producing Films - Melt Casting*

35

Weigh a known weight of PCL directly into a small glass beaker. Place the beaker in a larger beaker containing water (to act as a water bath) and put it on the hot plate at 70°C for 15 minutes or until the polymer has fully

- melted. Add a known weight of drug to the melted polymer and stir the mixture thoroughly. To aid dispersion of the drug in the melted PCL, the drug may be suspended/dissolved in a small volume (<10% of the volume of the melted PCL) of 100% ethanol. This ethanol suspension is then mixed into the melted polymer. Pour the melted polymer into a mould and let it to cool. After cooling, store the film in a container.

2. Procedure for Producing Films - Solvent Casting

- Weigh a known weight of PCL directly into a 20 ml glass scintillation vial and add sufficient DCM to achieve a 10% w/v solution. Cap the vial and mix the solution. Add sufficient taxol to the solution to achieve the desired final taxol concentration. Use hand shaking or vortexing to dissolve the taxol in the solution. Let the solution sit for one hour (to diminish the presence of air bubbles) and then pour it slowly into a mould. The mould used is based on the shape required. Place the mould in the fume hood overnight. This will allow the DCM to evaporate. Either leave the film in the mould to store it or peel it out and store it in a sealed container.

3. Procedure for Producing Films - Sprayed

- Weigh sufficient polymer directly into a 20 ml glass scintillation vial and add sufficient DCM to achieve a 2% w/v solution. Cap the vial and mix the solution to dissolve the polymer (hand shaking). Assemble the moulds in a vertical orientation in a suitable mould holding apparatus in the fume hood. Position this mould holding apparatus 6 to 12 inches above the fume hood floor on a suitable support (e.g., inverted 2000 ml glass beaker) to enable horizontal spraying. Using an automatic pipette, transfer a suitable volume (minimum 5 ml) of the 2% polymer solution to a separate 20 ml glass scintillation vial. Add sufficient taxol to the solution and dissolve it by hand shaking the capped vial. To prepare for spraying, remove the cap of this vial and dip the barrel (only) of an TLC atomizer into the polymer solution. Note: the reservoir of the atomizer is not used in this procedure - the 20 ml glass vial acts as a reservoir.

- Connect the nitrogen tank to the gas inlet of the atomizer. Gradually increase the pressure until atomization and spraying begins. Note the pressure and use this pressure throughout the procedure. To spray the moulds use 5 second oscillating sprays with a 15 second dry time between sprays. During the dry time, finger crimp the gas line to avoid wastage of the spray. Spraying is continued until a suitable thickness of polymer is deposited on the

mould. The thickness is based on the request. Leave the sprayed films attached to the moulds and store in sealed containers.

E. Procedure for Producing Nanopaste

5

Nanopaste is a suspension of microspheres suspended in a hydrophilic gel. Within one aspect of the invention, the gel or paste can be smeared over tissue as a method of locating drug loaded microspheres close to the target tissue. Being water based, the paste will soon become diluted with
10 bodily fluids causing a decrease in the stickiness of the paste and a tendency of the microspheres to be deposited on nearby tissue. A pool of microsphere encapsulated drug is thereby located close to the target tissue.

Reagents and equipment which were utilized within these experiments include glass beakers, Carbopol 925 (pharmaceutical grade,
15 Goodyear Chemical Co.), distilled water, sodium hydroxide (1 M) in water solution, sodium hydroxide solution (5 M) in water solution, microspheres in the 0.1 μm to 3 μm size range suspended in water at 20% w/v (See previous).

1. *Preparation of 5% w/v Carbopol Gel*

20

Add a sufficient amount of carbopol to 1 M sodium hydroxide to achieve a 5% w/v solution. To dissolve the carbopol in the 1 M sodium hydroxide, allow the mixture to sit for approximately one hour. During this time period, stir the mixture using a glass rod. After one hour, take the pH of the mixture. A low pH indicates that the carbopol is not fully dissolved. The pH
25 you want to achieve is 7.4. Use 5 M sodium hydroxide to adjust the pH. This is accomplished by *slowly* adding drops of 5 M sodium hydroxide to the mixture, stirring the mixture and taking the pH of the mixture. It usually takes approximately one hour to adjust the pH to 7.4. Once a pH of 7.4 is achieved, cover the gel and let it sit for 2 to 3 hours. After this time period, check the pH
30 to ensure it is still at 7.4. If it has changed, adjust back to pH 7.4 using 5 M sodium hydroxide. Allow the gel to sit for a few hours to ensure the pH is stable at 7.4. Repeat the process until the desired pH is achieved and is stable. Label the container with the name of the gel and the date. The gel is to be used to make nanopaste within the next week.

35

2. Procedure for Producing Nanopaste

- Add sufficient 0.1 μm to 3 μm microspheres to water to produce a 20% suspension of the microspheres. Put 8 ml of the 5% w/v carbopol gel in a glass beaker. Add 2 ml of the 20% microsphere suspension to the beaker.
- 5 Using a glass rod or a mixing spatula, stir the mixture to thoroughly disperse the microspheres throughout the gel. This usually takes 30 minutes. Once the microspheres are dispersed in the gel, place the mixture in a storage jar. Store the jar at 4°C. It must be used within a one month period.

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EXAMPLE 11

CONTROLLED DELIVERY OF TAXOL FROM MICROSPHERES COMPOSED OF A BLEND OF ETHYLENE-VINYL-ACETATE COPOLYMER AND POLY (D,L LACTIC ACID). IN VIVO TESTING OF THE MICROSPHERES ON THE CAM ASSAY

15

- This example describes the preparation of taxol-loaded microspheres composed of a blend of biodegradable poly (d,l-lactic acid) (PLA) polymer and nondegradable ethylene-vinyl acetate (EVA) copolymer. In addition, the *in vitro* release rate and anti-angiogenic activity of taxol released from microspheres placed on a CAM are demonstrated.
- 20

- Reagents which were utilized in these experiments include taxol, which is purchased from Sigma Chemical Co. (St. Louis, MO); PLA (molecular weight 15,000-25,000) and EVA (60% vinyl acetate) (purchased from Polysciences (Warrington, PA); polyvinyl alcohol (PVA) (molecular weight 25 124,000-186,000, 99% hydrolysed, purchased from Aldrich Chemical Co. (Milwaukee, WI)) and Dichloromethane (DCM) (HPLC grade, obtained from Fisher Scientific Co). Distilled water is used throughout.

30

A. Preparation of microspheres

- Microspheres are prepared essentially as described in Example 8 utilizing the solvent evaporation method. Briefly, 5% w/v polymer solutions in 20 mL DCM are prepared using blends of EVA:PLA between 35:65 to 90:10. To 5 mL of 2.5% w/v PVA in water in a 20 mL glass vial is added 1 mL of the polymer solution dropwise with stirring. Six similar vials are assembled in a six position overhead stirrer, dissolution testing apparatus (Vanderkamp) and stirred at 200 rpm. The temperature of the vials is increased from room
- 35

temperature to 40°C over 15 min and held at 40°C for 2 hours. Vials are centrifuged at 500xg and the microspheres washed three times in water. At some EVA:PLA polymer blends, the microsphere samples aggregated during the washing stage due to the removal of the dispersing or emulsifying agent, PVA. This aggregation effect could be analyzed semi-quantitatively since aggregated microspheres fused and the fused polymer mass floated on the surface of the wash water. This surface polymer layer is discarded during the wash treatments and the remaining, pelleted microspheres are weighed. The % aggregation is determined from

10

$$\% \text{ aggregation} = \frac{1 - (\text{weight of pelleted microspheres})}{\text{initial polymer weight}} \times 100$$

Taxol loaded microspheres (0.6% w/w taxol) are prepared by dissolving the taxol in the 5% w/v polymer solution in DCM. The polymer blend used is 50:50 EVA:PLA. A "large" size fraction and "small" size fraction of microspheres are produced by adding the taxol/polymer solution dropwise into 2.5% w/v PVA and 5% w/v PVA, respectively. The dispersions are stirred at 40°C at 200 rpm for 2 hours, centrifuged and washed 3 times in water as described previously. Microspheres are air dried and samples are sized using an optical microscope with a stage micrometer. Over 300 microspheres are counted per sample. Control microspheres (taxol absent) are prepared and sized as described previously.

25

B. Encapsulation efficiency

Known weights of taxol-loaded microspheres are dissolved in 1 mL DCM, 20 mL of 40% acetonitrile in water at 50°C are added and vortexed until the DCM had been evaporated. The concentration of taxol in the 40% acetonitrile is determined by HPLC using a mobile phase of water:methanol:acetonitrile (37:5:58) at a flow rate of 1 mL/min (Beckman isocratic pump), a C8 reverse phase column (Beckman) and UV detection at 232 nm. To determine the recovery efficiency of this extraction procedure, known weights of taxol from 100-1000 µg are dissolved in 1 mL of DCM and subjected to the same extraction procedure in triplicate as described previously. Recoveries are always greater than 85% and the values of encapsulation efficiency are corrected appropriately.

C. Drug release studies

5 In 15 mL glass, screw capped tubes are placed 10 mL of 10 mM
phosphate buffered saline (PBS), pH 7.4 and 35 mg taxol-loaded microspheres.
The tubes are tumbled at 37°C and at given time intervals, centrifuged at 1500xg
for 5 min and the supernatant saved for analysis. Microsphere pellets are
resuspended in fresh PBS (10mL) at 37°C and reincubated. Taxol
concentrations are determined by extraction into 1 mL DCM followed by
10 evaporation to dryness under a stream of nitrogen, reconstitution in 1 mL of
40% acetonitrile in water and analysis using HPLC as previously described.

D. Scanning Electron Microscopy (SEM)

15 Microspheres are placed on sample holders, sputter coated with
gold and micrographs obtained using a Philips 501B SEM operating at 15 kV.

E. CAM Studies

20 Fertilized, domestic chick embryos are incubated for 4 days prior
to shell-less culturing. The egg contents are incubated at 90% relative humidity
and 3% CO₂ for 2 days. On day 6 of incubation, 1 mg aliquots of 0.6% taxol
loaded or control (taxol free) microspheres are placed directly on the CAM
surface. After a 2 day exposure the vasculature is examined using a
25 stereomicroscope interfaced with a video camera; the video signals are then
displayed on a computer and video printed.

F. Results

30 Microspheres prepared from 100% EVA are freely suspended in
solutions of PVA but aggregated and coalesced or fused extensively on
subsequent washing in water to remove the PVA. Blending EVA with an
increasing proportion of PLA produced microspheres showing a decreased
tendency to aggregate and coalesce when washed in water, as described in
35 Figure 15A. A 50:50 blend of EVA:PLA formed microspheres with good
physical stability, that is the microspheres remained discrete and well suspended
with negligible aggregation and coalescence.

The size range for the "small" size fraction microspheres is determined to be >95% of the microsphere sample (by weight) between 10-30 mm and for the "large" size fraction, >95% of the sample (by weight) between 30-100 mm. Representative scanning electron micrographs of taxol loaded 50:50 EVA:PLA microspheres in the "small" and "large" size ranges are shown in Figures 15B and 15C, respectively. The microspheres are spherical with a smooth surface and with no evidence of solid drug on the surface of the microspheres. The efficiency of loading 50:50 EVA:PLA microspheres with taxol is between 95-100% at initial taxol concentrations of between 100-1000 mg taxol per 50 mg polymer. There is no significant difference (Student t-test, $p < 0.05$) between the encapsulation efficiencies for either "small" or "large" microspheres.

The time course of taxol release from 0.6% w/v loaded 50:50 EVA:PLA microspheres is shown in Figure 15D for "small" size (open circles) and "large" size (closed circles) microspheres. The release rate studies are carried out in triplicate tubes in 3 separate experiments. The release profiles are biphasic with an initial rapid release of taxol or "burst" phase occurring over the first 4 days from both size range microspheres. This is followed by a phase of much slower release. There is no significant difference between the release rates from "small" and "large" microspheres. Between 10-13% of the total taxol content of the microspheres is released in 50 days.

The taxol loaded microspheres (0.6% w/v loading) are tested using the CAM assay and the results are shown in Figure 15E. The taxol microspheres released sufficient drug to produce a zone of avascularity in the surrounding tissue (Figure 15F). Note that immediately adjacent to the microspheres ("MS" in Figures 15E and 15F) is an area in which blood vessels are completely absent (Zone 1); further from the microspheres is an area of disrupted, non-functioning capillaries (Zone 2); it is only at a distance of approximately 6 mm from the microspheres that the capillaries return to normal. In CAMs treated with control microspheres (taxol absent) there is a normal capillary network architecture.

Discussion

Arterial chemoembolization is a invasive surgical technique. Therefore, ideally, a chemoembolic formulation of an anti-angiogenic and anticancer drug such as taxol would release the drug at the tumor site at

concentrations sufficient for activity for a prolonged period of time, of the order of several months. EVA is a tissue compatible nondegradable polymer which has been used extensively for the controlled delivery of macromolecules over long time periods (> 100 days).

5 EVA is initially selected as a polymeric biomaterial for preparing microspheres with taxol dispersed in the polymer matrix. However, microspheres prepared with 100% EVA aggregated and coalesced almost completely during the washing procedure.

10 Polymers and copolymers based on lactic acid and glycolic acid are physiologically inert and biocompatible and degrade by hydrolysis to toxicologically acceptable products. Copolymers of lactic acid and glycolic acids have faster degradation rates than PLA and drug loaded microspheres prepared using these copolymers are unsuitable for prolonged, controlled release over several months. Dollinger and Sawan blended PLA with EVA and showed that
15 the degradation lifetime of PLA is increased as the proportion of EVA in the blend is increased. They suggested that blends of EVA and PLA should provide a polymer matrix with better mechanical stability and control of drug release rates than PLA.

Figure 15A shows that increasing the proportion of PLA in a
20 EVA:PLA blend decreased the extent of aggregation of the microsphere suspensions. Blends of 50% or less EVA in the EVA:PLA matrix produced physically stable microsphere suspensions in water or PBS. A blend of 50:50 EVA:PLA is selected for all subsequent studies.

Different size range fractions of microspheres could be prepared
25 by changing the concentration of the emulsifier, PVA, in the aqueous phase. "Small" microspheres are produced at the higher PVA concentration of 5% w/v whereas "large" microspheres are produced at 2.5% w/v PVA. All other production variables are the same for both microsphere size fractions. The higher concentration of emulsifier gave a more viscous aqueous dispersion
30 medium and produced smaller droplets of polymer/taxol/DCM emulsified in the aqueous phase and thus smaller microspheres. The taxol loaded microspheres contained between 95-100% of the initial taxol added to the organic phase encapsulated within the solid microspheres. The low water solubility of taxol favoured partitioning into the organic phase containing the
35 polymer.

Release rates of taxol from the 50:50 EVA:PLA microspheres are very slow with less than 15% of the loaded taxol being released in 50 days. The

initial burst phase of drug release may be due to diffusion of drug from the superficial region of the microspheres (close to the microsphere surface).

The mechanism of drug release from nondegradable polymeric matrices such as EVA is thought to involve the diffusion of water through the dispersed drug phase within the polymer, dissolution of the drug and diffusion of solute through a series of interconnecting, fluid filled pores. Blends of EVA and PLA have been shown to be immiscible or bicontinuous over a range of 30 to 70% EVA in PLA. In degradation studies in PBS buffer at 37°C, following an induction or lag period, PLA hydrolytically degraded and eroded from the EVA:PLA polymer blend matrix leaving an inactive sponge-like skeleton. Although the induction period and rate of PLA degradation and erosion from the blended matrices depended on the proportion of PLA in the matrix and on process history, there is consistently little or no loss of PLA until after 40-50 days.

Although some erosion of PLA from the 50:50 EVA:PLA microspheres may have occurred within the 50 days of the *in vitro* release rate study (Figure 15C), it is likely that the primary mechanism of drug release from the polymer blend is diffusion of solute through a pore network in the polymer matrix.

At the conclusion of the release rate study, the microspheres are analyzed from the amount of drug remaining. The values for the percent of taxol remaining in the 50 day incubation microsphere samples are 94% +/- 9% and 89% +/- 12% for "large" and "small" size fraction microspheres, respectively.

Microspheres loaded with 6mg per mg of polymer (0.6%) provided extensive inhibition of angiogenesis when placed on the CAM of the embryonic chick (Figures 15E and 15F).

30

EXAMPLE 12

TAXOL ENCAPSULATION IN POLY(E-CAPROLACTONE) MICROSPHERES. INHIBITION OF ANGIOGENESIS ON THE CAM ASSAY BY TAXOL-LOADED MICROSPHERES

This example evaluates the *in vitro* release rate profile of taxol from biodegradable microspheres of poly(e-caprolactone) and demonstrates the anti-angiogenic activity of taxol released from these microspheres when placed on the CAM.

Reagents which were utilized in these experiments include: poly(ϵ -caprolactone) ("PCL") (molecular weight 35,000 - 45,000; purchased from Polysciences (Warrington, PA)); dichloromethane ("DCM") from Fisher Scientific Co., Canada; polyvinyl alcohol (PVP) (molecular weight 12,000 - 18,000, 99% hydrolysed) from Aldrich Chemical Co. (Milwaukee, Wis.), and taxol from Sigma Chemical Co. (St. Louis, MO). Unless otherwise stated all chemicals and reagents are used as supplied. Distilled water is used throughout.

A. Preparation of microspheres

10

Microspheres are prepared essentially as described in Example 8 utilizing the solvent evaporation method. Briefly, 5%w/w taxol loaded microspheres are prepared by dissolving 10 mg of taxol and 190 mg of PCL in 2 ml of DCM, adding to 100 ml of 1% PVP aqueous solution and stirring at 1000 r.p.m. at 25°C for 2 hours. The suspension of microspheres is centrifuged at 1000 x g for 10 minutes (Beckman GPR), the supernatant removed and the microspheres washed three times with water. The washed microspheres are air-dried overnight and stored at room temperature. Control microspheres (taxol absent) are prepared as described above. Microspheres containing 1% and 2% taxol are also prepared. Microspheres are sized using an optical microscope with a stage micrometer.

B. Encapsulation efficiency

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A known weight of drug-loaded microspheres (about 5 mg) is dissolved in 8 ml of acetonitrile and 2 ml distilled water is added to precipitate the polymer. The mixture is centrifuged at 1000 g for 10 minutes and the amount of taxol encapsulated is calculated from the absorbance of the supernatant measured in a UV spectrophotometer (Hewlett-Packard 8452A Diode Array Spectrophotometer) at 232 nm.

C. Drug release studies

About 10 mg of taxol-loaded microspheres are suspended in 20 ml of 10 mM phosphate buffered saline, pH 7.4 (PBS) in screw-capped tubes. The tubes are tumbled end-over-end at 37°C and at given time intervals 19.5 ml of supernatant is removed (after allowing the microspheres to settle at the

bottom), filtered through a 0.45 mm membrane filter and retained for taxol analysis. An equal volume of PBS is replaced in each tube to maintain sink conditions throughout the study. The filtrates are extracted with 3 x 1 ml DCM, the DCM extracts evaporated to dryness under a stream of nitrogen, redissolved
5 in 1 ml acetonitrile and analyzed by HPLC using a mobile phase of water:methanol:acetonitrile (37:5:58) at a flow rate of 1ml min⁻¹ (Beckman Isocratic Pump), a C8 reverse phase column (Beckman), and UV detection (Shimadzu SPD A) at 232 nm.

10 D. CAM studies

Fertilized, domestic chick embryos are incubated for 4 days prior to shell-less culturing. On day 6 of incubation, 1 mg aliquots of 5% taxol-loaded or control (taxol-free) microspheres are placed directly on the CAM surface.
15 After a 2-day exposure the vasculature is examined using a stereomicroscope interfaced with a video camera; the video signals are then displayed on a computer and video printed.

20 E. Scanning electron microscopy

Microspheres are placed on sample holders, sputter-coated with gold and then placed in a Philips 501B Scanning Electron Microscope operating at 15 kV.

25 F. Results

The size range for the microsphere samples is between 30 - 100 mm, although there is evidence in all taxol-loaded or control microsphere batches of some microspheres falling outside this range. The efficiency of
30 loading PCL microspheres with taxol is always greater than 95% for all drug loadings studied. Scanning electron microscopy demonstrated that the microspheres are all spherical and many showed a rough or pitted surface morphology. There appeared to be no evidence of solid drug on the surface of the microspheres.

35 The time courses of taxol release from 1%, 2% and 5% loaded PCL microspheres are shown in Figure 16A. The release rate profiles are bi-phasic. There is an initial rapid release of taxol or "burst phase" at all drug

loadings. The burst phase occurred over 1-2 days at 1% and 2% taxol loading and over 3-4 days for 5% loaded microspheres. The initial phase of rapid release is followed by a phase of significantly slower drug release. For microspheres containing 1% or 2% taxol there is no further drug release after
5 21 days. At 5% taxol loading, the microspheres had released about 20% of the total drug content after 21 days.

Figure 16B shows CAMs treated with control PCL microspheres, and Figure 16C shows treatment with 5% taxol loaded microspheres. The CAM with the control microspheres shows a normal capillary network architecture.
10 The CAM treated with taxol-PCL microspheres shows marked vascular regression and zones which are devoid of a capillary network.

G. Discussion

15 The solvent evaporation method of manufacturing taxol-loaded microspheres produced very high taxol encapsulation efficiencies of between 95-100%. This is due to the poor water solubility of taxol and its hydrophobic nature favouring partitioning in the organic solvent phase containing the polymer.

20 The biphasic release profile for taxol is typical of the release pattern for many drugs from biodegradable polymer matrices. Poly(ϵ -caprolactone) is an aliphatic polyester which can be degraded by hydrolysis under physiological conditions and it is non-toxic and tissue compatible. The degradation of PCL is significantly slower than that of the extensively
25 investigated polymers and copolymers of lactic and glycolic acids and is therefore suitable for the design of long-term drug delivery systems. The initial rapid or burst phase of taxol release is thought to be due to diffusional release of the drug from the superficial region of the microspheres (close to the microsphere surface). Release of taxol in the second (slower) phase of the
30 release profiles is not likely due to degradation or erosion of PCL because studies have shown that under *in vitro* conditions in water there is no significant weight loss or surface erosion of PCL over a 7.5-week period. The slower phase of taxol release is probably due to dissolution of the drug within fluid-filled pores in the polymer matrix and diffusion through the pores. The greater
35 release rate at higher taxol loading is probably a result of a more extensive pore network within the polymer matrix.

Taxol microspheres with 5% loading have been shown to release sufficient drug to produce extensive inhibition of angiogenesis when placed on the CAM. The inhibition of blood vessel growth resulted in an avascular zone as shown in Figure 16C.

5

EXAMPLE 13

TAXOL-LOADED POLYMERIC FILMS COMPOSED OF ETHYLENE VINYL ACETATE AND A SURFACTANT

10

Two types of films are prepared essentially as described in Example 10: pure EVA films loaded with taxol and EVA/surfactant blend films (*i.e.*, Pluronic F127, Span 80 and Pluronic L101) loaded with taxol.

15 The surfactants being examined are two hydrophobic surfactants (Span 80 and Pluronic L101) and one hydrophilic surfactant (Pluronic F127). The pluroinc surfactants are themselves polymers, which is an attractive property since they can be blended with EVA to optimize various drug delivery properties. Span 80 is a smaller molecule which is in some manner dispersed in the polymer matrix, and does not form a blend.

20 Surfactants will be useful in modulating the release rates of taxol from films and optimizing certain physical parameters of the films. One aspect of the surfactant blend films which indicates that drug release rates can be controlled is the ability to vary the rate and extent to which the compound will swell in water. Diffusion of water into a polymer-drug matrix is critical to the
25 release of drug from the carrier. Figures 17C and 17D show the degree of swelling of the films as the level of surfactant in the blend is altered. Pure EVA films do not swell to any significant extent in over 2 months. However, by increasing the level of surfactant added to the EVA it is possible to increase the degree of swelling of the compound, and by increasing hydrophilicity swelling
30 can also be increased.

Results of experiments with these films are shown below in Figures 17A-E. Briefly, Figure 17A shows taxol release (in mg) over time from pure EVA films. Figure 17B shows the percentage of drug remaining for the same films. As can be seen from these two figures, as taxol loading increases
35 (*i.e.*, percentage of taxol by weight is increased), drug release rates increase, showing the expected concentration dependence. As taxol loading is increased,

the percent taxol remaining in the film also increases, indicating that higher loading may be more attractive for long-term release formulations.

Physical strength and elasticity of the films is assessed in Figure 17E. Briefly, Figure 17E shows stress/strain curves for pure EVA and EVA-Surfactant blend films. This crude measurement of stress demonstrates that the elasticity of films is increased with the addition of Pluronic F127, and that the tensile strength (stress on breaking) is increased in a concentration dependant manner with the addition of Pluronic F127. Elasticity and strength are important considerations in designing a film which can be manipulated for particular clinical applications without causing permanent deformation of the compound.

The above data demonstrates the ability of certain surfactant additives to control drug release rates and to alter the physical characteristics of the vehicle.

EXAMPLE 14

INCORPORATING METHOXYPOLYETHYLENE GLYCOL 350 (MePEG) INTO POLY(E-CAPROLACTONE) TO DEVELOP A FORMULATION FOR THE CONTROLLED DELIVERY OF TAXOL FROM A PASTE

Reagents and equipment which were utilized within these experiments include methoxypolyethylene glycol 350 ("MePEG" - Union Carbide, Danbury, CT). MePEG is liquid at room temperature, and has a freezing point of 10° to -5°C.

A. Preparation of a MePEG/PCL taxol-containing paste

MePEG/PCL paste is prepared by first dissolving a quantity of taxol into MePEG, and then incorporating this into melted PCL. One advantage with this method is that no DCM is required.

B. Analysis of melting point

The melting point of PCL/MePEG polymer blends may be determined by differential scanning calorimetry from 30°C to 70°C at a heating rate of 2.5°C per minute. Results of this experiment are shown in Figures 18A

and 18B. Briefly, as shown in Figure 18A the melting point of the polymer blend (as determined by thermal analysis) is decreased by MePEG in a concentration dependent manner. The melting point of the polymer blends as a function of MePEG concentration is shown in Figure 18A. This lower melting point also translates into an increased time for the polymer blends to solidify from melt as shown in Figure 18B. A 30:70 blend of MePEG:PCL takes more than twice as long to solidify from the fluid melt than does PCL alone.

C. Measurement of brittleness

10

Incorporation of MePEG into PCL appears to produce a less brittle solid, as compared to PCL alone. As a "rough" way of quantitating this, a weighted needle is dropped from an equal height into polymer blends containing from 0% to 30% MePEG in PCL, and the distance that the needle penetrates into the solid is then measured. The resulting graph is shown as Figure 18C. Points are given as the average of four measurements ± 1 S.D.

15

For purposes of comparison, a sample of paraffin wax is also tested and the needle penetrated into this a distance of 7.25 mm \pm 0.3 mm.

20

D. Measurement of taxol release

Pellets of polymer (PCL containing 0%, 5%, 10% or 20% MePEG) are incubated in phosphate buffered saline (PBS, pH 7.4) at 37°C, and % change in polymer weight is measured over time. As can be seen in Figure 18D, the amount of weight lost increases with the concentration of MePEG originally present in the blend. It is likely that this weight loss is due to the release of MePEG from the polymer matrix into the incubating fluid. This would indicate that taxol will readily be released from a MePEG/PCL blend since taxol is first dissolved in MePEG before incorporation into PCL.

30

E. Effect of varying quantities of MePEG on taxol release

Thermopastes are made up containing between 0.8% and 20% MePEG in PCL. These are loaded with 1% taxol. The release of taxol over time from 10 mg pellets in PBS buffer at 37°C is monitored using HPLC. As is shown in Figure 18E, the amount of MePEG in the formulation does not affect the amount of taxol that is released.

35

F. Effect of varying quantities of taxol on the total amount of taxol released from a 20% MePEG/PCL blend

5 Thermopastes are made up containing 20% MePEG in PCL and loaded with between 0.2% and 10% taxol. The release of taxol over time is measured as described above. As shown in Figure 18F, the amount of taxol released over time increases with increased taxol loading. When plotted as the percent total taxol released, however, the order is reversed (Figure 18G). This
10 gives information about the residual taxol remaining in the paste and, if assumptions are made about the validity of extrapolating this data, allows for a projection of the period of time over which taxol will be released from the 20% MePEG Thermopaste.

15 G. Strength analysis of various MePEG/PCL blends

A CT-40 mechanical strength tester is used to measure the strength of solid polymer "tablets" of diameter 0.88 cm and an average thickness of 0.560 cm. The polymer tablets are blends of MePEG at concentrations of
20 0%, 5%, 10% or 20% in PCL.

Results of this test are shown in Figure 18H, where both the tensile strength and the time to failure are plotted as a function of %MePEG in the blend. Single variable ANOVA indicated that the tablet thicknesses within each group are not different. As can be seen from Figure 18H, the addition of
25 MePEG into PCL decreased the hardness of the resulting solid.

EXAMPLE 15

EFFECT OF TAXOL-LOADED THERMOPASTE ON
30 ANGIOGENESIS *IN VIVO*

Fertilized, domestic chick embryos were incubated for 4 days prior to shell-less culturing as described in Example 2. The egg contents are removed from the shell and emptied into round-bottom sterilized glass bowls
35 and covered with petri dish covers.

Taxol is incorporated into thermopaste at concentrations of 5%, 10%, and 20% (w/v) essentially as described above (see Example 10), and used

in the following experiments. Dried cut thermopaste is then heated to 60°C and pressed between two sheets of parafilm, flattening it, and allowing it to cool. Six embryos received 20% taxol-loaded thermopaste and 6 embryos received unloaded thermopaste prepared in this manner. One embryo died in each group leaving 5 embryos in each of the control and treated groups.

Unloaded thermopaste and thermopaste containing 20% taxol was also heated to 60°C and placed directly on the growing edge of each CAM at day 6 of incubation; two embryos each were treated in this manner.

There was no observable difference in the results obtained using the different methods of administration, indicating that the temperature of the paste at the time of application was not a factor in the outcome.

Thermopaste with 10% taxol was applied to 11 CAMs and unloaded thermopaste was applied to an additional 11 CAMs, while 5% taxol-loaded thermopaste was applied to 10 CAMs and unloaded thermopaste was applied to 10 other control CAMs. After a 2 day exposure (day 8 of incubation) the vasculature was examined with the aid of a stereomicroscope. Liposyn II, a white opaque solution, was injected into the CAM to increase the visibility of the vascular details.

In the embryos treated with 5% taxol-loaded paste, only 2 animals demonstrated maximum inhibition of angiogenesis, while the remaining 8 were only marginally affected. Of the animals treated with 10% taxol-loaded thermopaste only 2 showed maximal inhibition while the other 9 were only marginally affected.

The 20% taxol-loaded thermopaste showed extensive areas of avascularity (see Figure 19B) in all 5 of the CAMs receiving this treatment. The highest degree of inhibition was defined as a region of avascularity covering 6 mm by 6 mm in size. All of the CAMs treated with 20% taxol-loaded thermopaste displayed this degree of angiogenesis inhibition.

By comparison, the control (unloaded) thermopaste did not inhibit angiogenesis on the CAM (see Figure 19A); this higher magnification view (note that the edge of the paste is seen at the top of the image) demonstrates that the vessels adjacent to the paste are unaffected by the thermopaste. This suggests that the effect observed is due to the sustained release of taxol and is not due to the polymer itself or due to a secondary pressure effect of the paste on the developing vasculature.

This study demonstrates that thermopaste releases sufficient quantities of angiogenesis inhibitor (in this case taxol) to inhibit the normal development of the CAM vasculature.

5

EXAMPLE 16

EFFECT OF TAXOL-LOADED THERMOPASTE ON TUMOR GROWTH AND TUMOR ANGIOGENESIS *In Vivo*

10 Fertilized domestic chick embryos are incubated for 3 days prior to having their shells removed. The egg contents are emptied by removing the shell located around the airspace, severing the interior shell membrane, perforating the opposite end of the shell and allowing the egg contents to gently slide out from the blunted end. The contents are emptied into round-bottom
15 sterilized glass bowls, covered with petri dish covers and incubated at 90% relative humidity and 3% carbon dioxide (see Example 2).

MDAY-D2 cells (a murine lymphoid tumor) is injected into mice and allowed to grow into tumors weighing 0.5-1.0 g. The mice are sacrificed, the tumor sites wiped with alcohol, excised, placed in sterile tissue culture media,
20 and diced into 1 mm pieces under a laminar flow hood. Prior to placing the dissected tumors onto the 9-day old chick embryos, CAM surfaces are gently scraped with a 30 gauge needle to insure tumor implantation. The tumors are then placed on the CAMs after 8 days of incubation (4 days after deshelling), and allowed to grow on the CAM for four days to establish a vascular supply.
25 Four embryos are prepared utilizing this method, each embryo receiving 3 tumors. For these embryos, one tumor receives 20% taxol-loaded thermopaste, the second tumor unloaded thermopaste, and the third tumor no treatment. The treatments are continued for two days before the results were recorded.

The explanted MDAY-D2 tumors secrete angiogenic factors
30 which induce the ingrowth of capillaries (derived from the CAM) into the tumor mass and allow it to continue to grow in size. Since all the vessels of the tumor are derived from the CAM, while all the tumor cells are derived from the explant, it is possible to assess the effect of therapeutic interventions on these two processes independently. This assay has been used to determine the
35 effectiveness of taxol-loaded thermopaste on: (a) inhibiting the vascularization of the tumor and (b) inhibiting the growth of the tumor cells themselves.

Direct *in vivo* stereomicroscopic evaluation and histological examination of fixed tissues from this study demonstrated the following. In the tumors treated with 20% taxol-loaded thermopaste, there was a reduction in the number of the blood vessels which supplied the tumor (see Figures 20C and 20D), a reduction in the number of blood vessels within the tumor, and a reduction in the number of blood vessels in the periphery of the tumor (the area which is typically the most highly vascularized in a solid tumor) when compared to control tumors. The tumors began to decrease in size and mass during the two days the study was conducted. Additionally, numerous endothelial cells were seen to be arrested in cell division indicating that endothelial cell proliferation had been affected. Tumor cells were also frequently seen arrested in mitosis. All 4 embryos showed a consistent pattern with the 20% taxol-loaded thermopaste suppressing tumor vascularity while the unloaded thermopaste had no effect.

By comparison, in CAMs treated with unloaded thermopaste, the tumors were well vascularized with an increase in the number and density of vessels when compared to that of the normal surrounding tissue, and dramatically more vessels than were observed in the tumors treated with taxol-loaded paste. The newly formed vessels entered the tumor from all angles appearing like spokes attached to the center of a wheel (see Figures 20A and 20B). The control tumors continued to increase in size and mass during the course of the study. Histologically, numerous dilated thin-walled capillaries were seen in the periphery of the tumor and few endothelial were seen to be in cell division. The tumor tissue was well vascularized and viable throughout.

As an example, in two similarly-sized (initially, at the time of explantation) tumors placed on the same CAM the following data was obtained. For the tumor treated with 20% taxol-loaded thermopaste the tumor measured 330 mm x 597 mm; the immediate periphery of the tumor has 14 blood vessels, while the tumor mass has only 3-4 small capillaries. For the tumor treated with unloaded thermopaste the tumor size was 623 mm x 678 mm; the immediate periphery of the tumor has 54 blood vessels, while the tumor mass has 12-14 small blood vessels. In addition, the surrounding CAM itself contained many more blood vessels as compared to the area surrounding the taxol-treated tumor.

This study demonstrates that thermopaste releases sufficient quantities of angiogenesis inhibitor (in this case taxol) to inhibit the pathological angiogenesis which accompanies tumor growth and development. Under these

conditions angiogenesis is maximally stimulated by the tumor cells which produce angiogenic factors capable of inducing the ingrowth of capillaries from the surrounding tissue into the tumor mass. The 20% taxol-loaded thermopaste is capable of blocking this process and limiting the ability of the tumor tissue to maintain an adequate blood supply. This results in a decrease in the tumor mass both through a cytotoxic effect of the drug on the tumor cells themselves and by depriving the tissue of the nutrients required for growth and expansion.

10

EXAMPLE 17

EFFECT OF ANGIOGENESIS INHIBITOR-LOADED THERMOPASTE ON TUMOR GROWTH *In Vivo* IN A MURINE TUMOR MODEL

The murine MDAY-D2 tumor model may be used to examine the effect of local slow release of the chemotherapeutic and anti-angiogenic compounds such as taxol on tumor growth, tumor metastasis, and animal survival. The MDAY-D2 tumor cell line is grown in a cell suspension consisting of 5% Fetal Calf Serum in alpha mem media. The cells are incubated at 37°C in a humidified atmosphere supplemented with 5% carbon dioxide, and are diluted by a factor of 15 every 3 days until a sufficient number of cells are obtained. Following the incubation period the cells are examined by light microscopy for viability and then are centrifuged at 1500 rpm for 5 minutes. PBS is added to the cells to achieve a dilution of 1,000,000 cells per ml.

Ten week old DBA/2j female mice are acclimatized for 3-4 days after arrival. Each mouse is then injected subcutaneously in the posteriolateral flank with 100,000 MDAY-D2 cells in 100 ml of PBS. Previous studies have shown that this procedure produces a visible tumor at the injection site in 3-4 days, reach a size of 1.0-1.7g by 14 days, and produces visible metastases in the liver 19-25 days post-injection. Depending upon the objective of the study a therapeutic intervention can be instituted at any point in the progression of the disease.

Using the above animal model, 20 mice are injected with 140,000 MDAY-D2 cells s.c. and the tumors allowed to grow. On day 5 the mice are divided into groups of 5. The tumor site was surgically opened under anesthesia, the local region treated with the drug-loaded thermopaste or control thermopaste without disturbing the existing tumor tissue, and the wound was closed. The groups of 5 received either no treatment (wound merely closed),

polymer (PCL) alone, 10% taxol-loaded thermopaste, or 20% taxol-loaded thermopaste (only 4 animals injected) implanted adjacent to the tumor site. On day 16, the mice were sacrificed, the tumors were dissected and examined (grossly and histologically) for tumor growth, tumor metastasis, local and systemic toxicity resulting from the treatment, effect on wound healing, effect on tumor vascularity, and condition of the paste remaining at the incision site.

The weights of the tumors for each animal is shown in the table below:

10

Table IV
Tumor Weights (gm)

	Animal No.	Control (empty)	Control (PCL)	10% Taxol Thermopaste	20% Taxol Thermopaste
15					
	1	1.387	1.137	0.487	0.114
	2	0.589	0.763	0.589	0.192
	3	0.461	0.525	0.447	0.071
	4	0.606	0.282	0.274	0.042
20	5	0.353	0.277	0.362	
	Mean	0.6808	0.6040	0.4318	0.1048
	Std. Deviation	0.4078	0.3761	0.1202	0.0653
25	P Value		0.7647	0.358	0.036

Thermopaste loaded with 20% taxol reduced tumor growth by over 85% (average weight 0.105) as compared to control animals (average weight 0.681). Animals treated with thermopaste alone or thermopaste containing 10% taxol had only modest effects on tumor growth; tumor weights were reduced by only 10% and 35% respectively (Figure 21A). Therefore, thermopaste containing 20% taxol was more effective in reducing tumor growth than thermopaste containing 10% taxol (see Figure 21C; see also Figure 21B).

Thermopaste was detected in some of the animals at the site of administration. Polymer varying in weight between 0.026 g to 0.078 g was detected in 8 of 15 mice. Every animal in the group containing 20% taxol-loaded thermopaste contained some residual polymer suggesting that it was less susceptible to dissolution. Histologically, the tumors treated with taxol-loaded

thermopaste contained lower cellularity and more tissue necrosis than control tumors. The vasculature was reduced and endothelial cells were frequently seen to be arrested in cell division. The taxol-loaded thermopaste did not appear to affect the integrity or cellularity of the skin or tissues surrounding the tumor.

5 Grossly, wound healing was unaffected.

EXAMPLE 18

10 THE USE OF ANGIOGENESIS-INHIBITOR LOADED SURGICAL FILMS IN THE PREVENTION OF IATROGENIC METASTATIC SEEDING OF TUMOR CELLS DURING CANCER RESECTION SURGERY

As a sterile, pliable, stretchable drug-polymer compound would be useful during cancer resection procedures. Often it is desirable to isolate the

15 normal surrounding tissues from malignant tissue during resection operations to prevent iatrogenic spread of the disease to adjacent organs through inadvertent contamination by cancer cells. A drug-loaded parafilm could be stretched across normal tissues prior to manipulation of the tumor. This would be most useful if placed around the liver and other abdominal contents during bowel

20 cancer resection surgery to prevent intraperitoneal spread of the disease to the liver. A biodegradable film could be left *in situ* to provide continued protection.

Incision sites are also a common location of post-operative recurrence of malignancy. This is thought to be due to contamination of the wound site with tumor cells during the surgical procedure. To address these

25 issues, experiments are being conducted to determine the ability of angiogenesis inhibitor-loaded films to prevent this phenomenon.

A. Materials and Methods

30 Preparation of Surgical Film. Surgical films are prepared as described in Example 10. Thin films measuring approximately 1 cm x 1 cm are prepared containing either polymer alone (PCL) or PCL loaded with 5% taxol.

Rat Hepatic Tumor Model. In an initial study Wistar rats weighing

35 approximately 300 g underwent general anesthesia and a 3-5 cm abdominal incision is made along the midline. In the largest hepatic lobe, a 1 cm incision is made in the hepatic parenchyma and part of the liver edge is resected. A

concentration of 1 million live 9L Glioma tumor cells (eluted from tissue culture immediately prior to the procedure) suspended in 100 ml of phosphate buffered saline are deposited onto the cut liver edge with a 30 gauge needle. The surgical is then placed over the cut liver edge containing the tumor cells and
5 affixed in place with Gelfoam. Two animals received PCL films containing 5% taxol and two animals received films containing PCL alone. The abdominal wall is closed with 3.0 Dexon and skin clips. The general anesthetic is terminated and the animal is allowed to recover. Ten days later the animals are sacrificed and the livers examined histologically.

10

B. Results

Local tumour growth is seen in the 2 livers treated with polymer alone. Both livers treated with polymer plus taxol are completely free of tumour
15 when examined histologically. Also of importance, the liver capsule had regenerated and grown completely over the polymeric film and the cut surface of the liver indicating that there is no significant effect on wound healing. There is no evidence of local hepatic toxicity surrounding any (drug-loaded or drug-free) of the surgical films.

20

C. Discussion

This study indicates that surgical films placed around normal tissues and incision sites during surgery may be capable of decreasing the
25 incidence of accidental implantation of tumor cells into normal surrounding tissue during resection of malignant tumors. This may help reduce the incidence of the significant problem of post-operative local recurrence of the disease.

30

EXAMPLE 19

INTRA-ARTICULAR INJECTION OF ANGIOGENESIS-INHIBITOR-LOADED BIODEGRADABLE MICROSpheres IN THE TREATMENT OF ARTHRITIS

35

Articular damage in arthritis is due to a combination of inflammation (including WBCs and WBC products) and pannus tissue development (a tissue composed on neovascular tissue, connective tissue, and inflammatory cells). Taxol has been chosen for the initial studies because it is a

potent inhibitor of neovascularization. In this manner, taxol in high local concentrations will prove to be a disease modifying agent in arthritis.

In order to determine whether microspheres have a deleterious effect on joints, the following experiments are conducted. Briefly, plain PCL
5 and taxol-loaded microspheres are prepared as described previously in Example 8.

Three rabbits are injected intra-articularly with 0.5-5.0 μm , 10-30 μm , or 30-80 μm microspheres in a total volume of 0.2 mls (containing 0.5 mg of microspheres). The joints are assessed visually (clinically) on a daily basis.
10 After two weeks the animals are sacrificed and the joints examined histologically for evidence of inflammation and depletion of proteoglycans.

The rabbit inflammatory arthritis and osteoarthritis models are being used to evaluate the use of microspheres in reducing synovitis and cartilage degradation. Degenerative arthritis is induced by a partial tear of the
15 cruciate ligament and meniscus of the knee. After 4 to 6 weeks, the rabbits develop erosions in the cartilage similar to that observed in human osteoarthritis. Inflammatory arthritis is induced by immunizing rabbits with bovine serum albumen (BSA) in Complete Freund's Adjuvant (CFA). After 3 weeks, rabbits containing a high titer of anti-BSA antibody receive an intra-
20 articular injection of BSA (5 mg). Joint swelling and pronounced synovitis is apparent by seven days, a proteoglycan depletion is observed by 7 to 14 days, and cartilage erosions are observed by 4 to 6 weeks.

Inflammatory arthritis is induced as described above. After 4 days, the joints are injected with microspheres containing 5% taxol or vehicle.
25 One group of animals will be sacrificed on day 14 and another on day 28. The joints are examined histologically for inflammation and cartilage degradation. The experiment is designed to determine if taxol microspheres can affect joint inflammation and cartilage matrix degradation.

Angiogenesis-inhibitor microspheres may be further examined in
30 an osteoarthritis model. Briefly, degenerative arthritis is induced in rabbits as described above, and the joints receive an intra-articular injection of microspheres (5% taxol or vehicle only) on day 4. The animals are sacrificed on day 21 and day 42 and the joints examined histologically for evidence of cartilage degradation.

35 Studies are conducted to assess angiogenesis inhibitors delivered via intra-articular microspheres as chondroprotective agents.

Results

Unloaded PCL microspheres of differing sizes (0.5-5.0 μm , 10-30 μm , or 30-80 μm) were injected intra-articularly into the rabbit knee joint. Results of these experiments are shown in Figures 22A to D. Briefly, Figure 22A is a photograph of synovium from PBS injected joints. Figure 22B is a photograph of joints injected with microspheres. Figure 22C is a photograph of cartilage from joints injected with PBS, and Figure 22D is a photograph of cartilage from joints injected with microspheres.

As can be seen from these photographs, histologically, there is no difference between joints receiving a microsphere injection and those which did not. Clinically, there was no evidence of joint inflammation during the 14 days the experiment was conducted. Grossly, there is no evidence of joint inflammation or cartilage damage in joints where microspheres are injected, as compared to untreated normal joints.

Conclusions

Microspheres can be injected intra-articularly without causing any discernible changes to the joint surface. This indicates that this method may be an effective means of delivering a targeted, sustained-release of disease-modifying agents to diseased joints, while minimizing the toxicity which could be associated with the systemic administration of such biologically active compounds.

As discussed above, microspheres can be formulated into specific sizes with defined drug release kinetics. It has also been demonstrated that taxol is a potent inhibitor of angiogenesis and that it is released from microspheres in quantities sufficient to block neovascularization on the CAM assay. Therefore, intra-articular administration of angiogenesis-inhibitor-loaded (e.g., taxol-loaded) microspheres should be capable of blocking the neovascularization that occurs in diseases such as rheumatoid arthritis and leads to cartilage destruction in the joint. In this manner the drug-loaded microspheres can act as a "chondroprotective" agent which protects the cartilage from irreversible destruction from invading neovascular pannus tissue.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of

illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Pro Gly Tyr Ile Gly Ser Arg

1

5

Claims

1. A composition, comprising:
 - (a) an anti-angiogenic factor; and
 - (b) a polymeric carrier.
2. The composition of claim 1 wherein said anti-angiogenic factor is Anti-Invasive Factor.
3. The composition of claim 1 wherein said anti-angiogenic factor is retinoic acid and derivatives thereof.
4. The composition of claim 1 wherein said anti-angiogenic factor is selected from the group consisting of Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1 and Plasminogen Activator Inhibitor-2.
5. A composition, comprising:
 - (a) taxol; and
 - (b) a polymeric carrier.
6. The composition according to any one of claims 1 to 5 wherein said composition is formed into microspheres having an average size of between 0.1 and 200 μm .
7. The composition according to any one of claims 1 to 5 wherein said composition is formed into a film with a thickness of between 100 μm and 2 mm.
8. The composition according to any one of claims 1 to 5 wherein said composition is liquid above 45°C, and solid or semi-solid at 37°C.
9. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is poly(ethylene-vinyl acetate) crosslinked with 40% vinyl acetate.
10. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is poly(lactic-co-glycolic acid).

11. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is polycaprolactone.

12. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is polylactic acid.

13. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is a copolymer of poly(ethylene-vinyl acetate) crosslinked with 40% vinyl acetate, and polylactic acid.

14. The composition according to any one of claim 1 to 5 wherein said polymeric carrier is a copolymer of polylactic acid and polycaprolactone.

15. A method for embolizing a blood vessel, comprising delivering into said vessel a therapeutically effective amount of composition according to any one of claims 1-14, such that said blood vessel is effectively occluded.

16. The method claim 13 wherein said blood vessel nourishes a tumor.

17. A stent, comprising a generally tubular structure, the surface of which is coated with a composition according to any one of claims 1-14.

18. A method for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of said structure being coated with a composition according to any one of claims 1-14, such that said passageway is expanded.

19. A method for eliminating vascular obstructions, comprising inserting a vascular stent into a vascular passageway, the stent having a generally tubular structure, the surface of said structure being coated with a composition according to any one of claims 1-14, such that said vascular obstruction is eliminated.

20. A method for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway, the stent having a generally tubular

structure, the surface of said structure being coated with a composition according to any one of claims 1-14, such that said biliary obstruction is eliminated.

21. A method for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra, the stent having a generally tubular structure, the surface of said structure being coated with a composition according to any one of claims 1-14, such that said urethral obstruction is eliminated.

22. A method for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus, the stent having a generally tubular structure, the surface of said structure being coated with a composition according to any one of claims 1-14, such that said esophageal obstruction is eliminated.

23. A method for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi, the stent having a generally tubular structure, the surface of which is coated with a composition according to any one of claims 1-14, such that said tracheal/bronchial obstruction is eliminated.

24. A method for treating a tumor excision site, comprising administering a composition according to any one of claims 1-14 to the resection margin of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at said site is inhibited.

25. The method according to claim 24 wherein said anti-angiogenic composition is a thermopaste.

26. The method according to claim 24 wherein the step of administering comprising spraying a composition of nanospheres comprised of an anti-angiogenic composition into the resection margin of the tumor.

27. A method for treating corneal neovascularization, comprising administering a therapeutically effective amount of a composition according to any one of claims 1-14 to the cornea, such that the formation of blood vessels is inhibited.

28. The method of claim 23 wherein said composition further comprises a topical corticosteroid.

29. A method for inhibiting angiogenesis in patients with non-tumorigenic, angiogenesis-dependent diseases, comprising administering a therapeutically effective amount of a composition comprising taxol to a patient with a non-tumorigenic angiogenesis-dependent disease, such that the formation of new blood vessels is inhibited.

30. A method for embolizing a blood vessel in a non-tumorigenic, angiogenesis-dependent diseases, comprising delivering to said vessel a therapeutically effective amount of a composition comprising taxol, such that said blood vessel is effectively occluded.

31. A method for expanding the lumen of a body passageway, comprising inserting a stent into the passageway, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said passageway is expanded.

32. A method for eliminating vascular obstructions, comprising inserting a vascular stent into a vascular passageway, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said vascular obstruction is eliminated.

33. A method for eliminating biliary obstructions, comprising inserting a biliary stent into a biliary passageway, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said biliary obstruction is eliminated.

34. A method for eliminating urethral obstructions, comprising inserting a urethral stent into a urethra, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said urethral obstruction is eliminated.

35. A method for eliminating esophageal obstructions, comprising inserting an esophageal stent into an esophagus, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said esophageal obstruction is eliminated.

36. A method for eliminating tracheal/bronchial obstructions, comprising inserting a tracheal/bronchial stent into the trachea or bronchi, the stent having a generally tubular structure, the surface of said structure being coated with a composition comprising taxol, such that said tracheal/bronchial obstruction is eliminated.

37. A method for treating a tumor excision site, comprising administering a composition comprising taxol to the resection margin of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at said site is inhibited.

38. A method for treating corneal neovascularization, comprising administering a therapeutically effective amount of a composition comprising taxol to the cornea, such that the formation of new vessels is inhibited.

39. A pharmaceutical product, comprising:
 (a) taxol, in a container; and
 (b) a notice associated with said container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by said agency of said taxol, for human or veterinary administration to treat non-tumorigenic angiogenesis-dependent diseases.

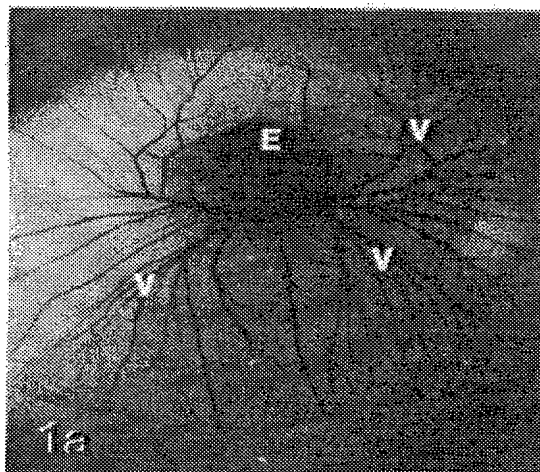


FIG. 1A



FIG. 1B

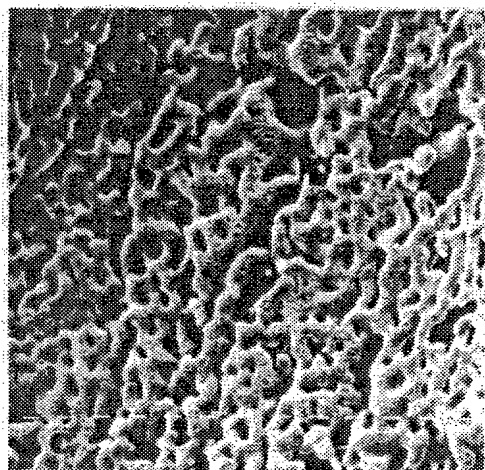
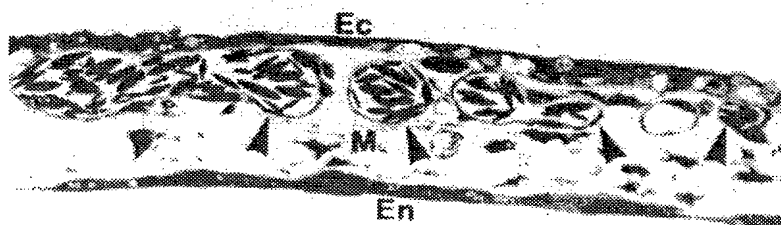


FIG. 1C

FIG. 1D



d

SUBSTITUTE SHEET

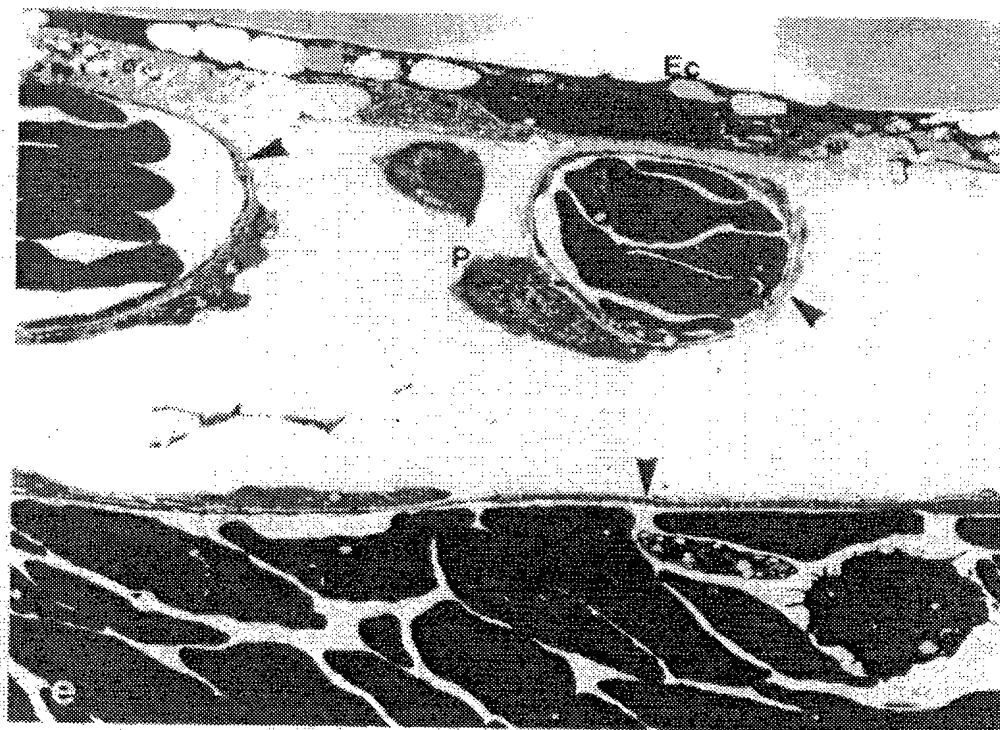
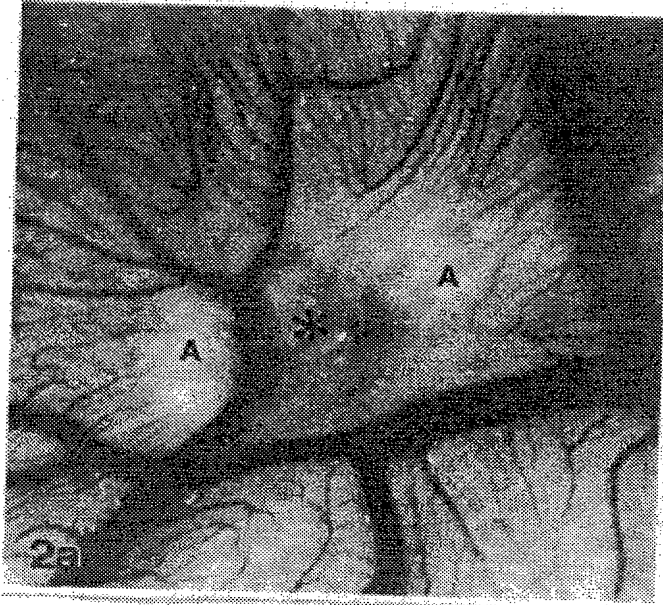
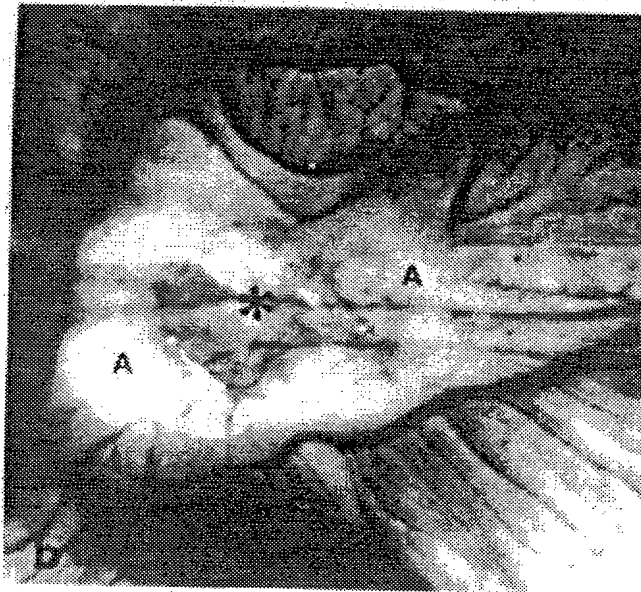
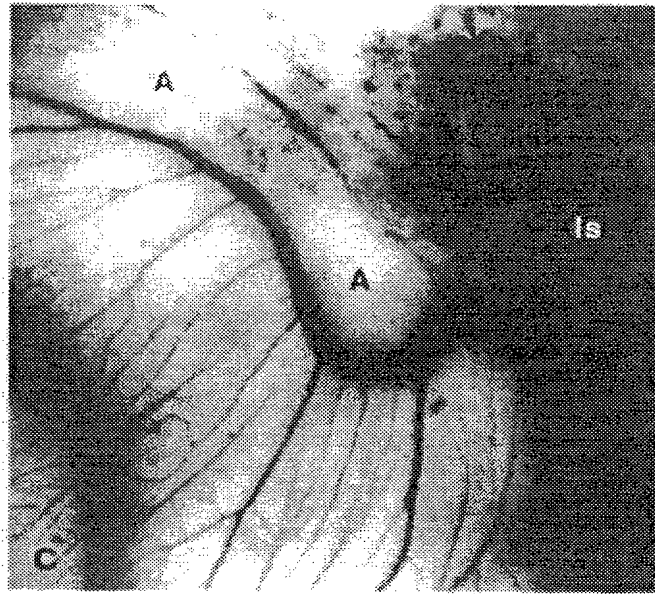
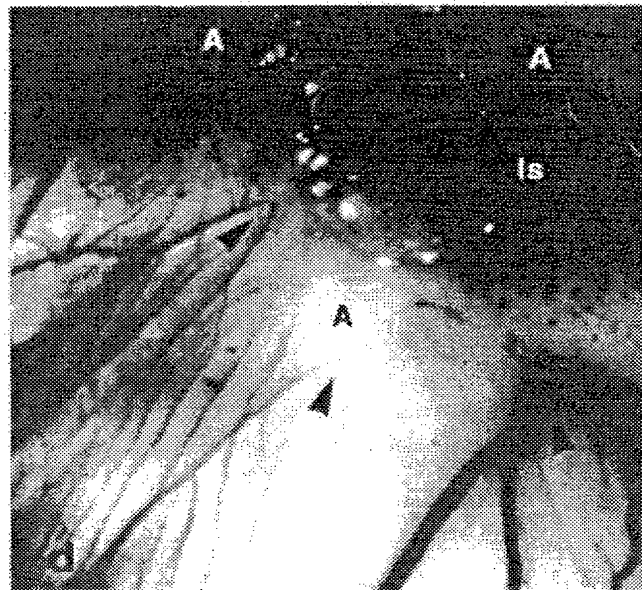


FIG. 1E

*FIG. 2A**FIG. 2B*

SUBSTITUTE SHEET

*FIG. 2C**FIG. 2D*

SUBSTITUTE SHEET

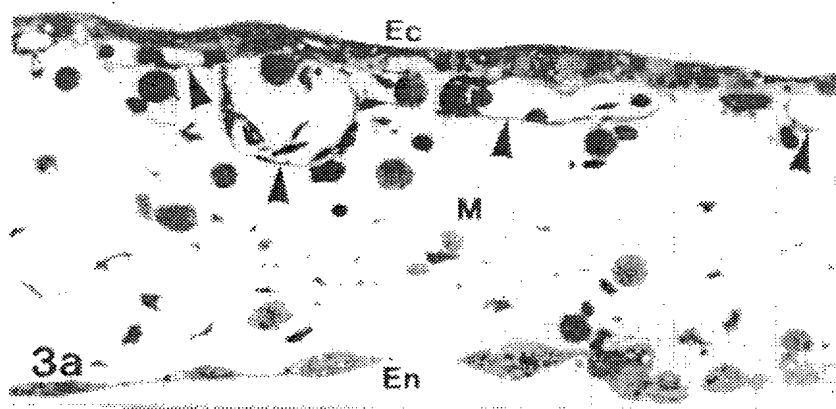


FIG. 3A

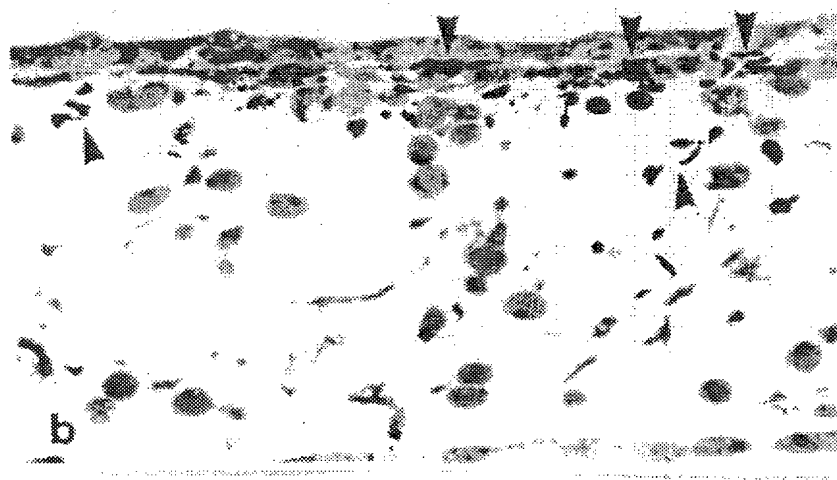


FIG. 3B

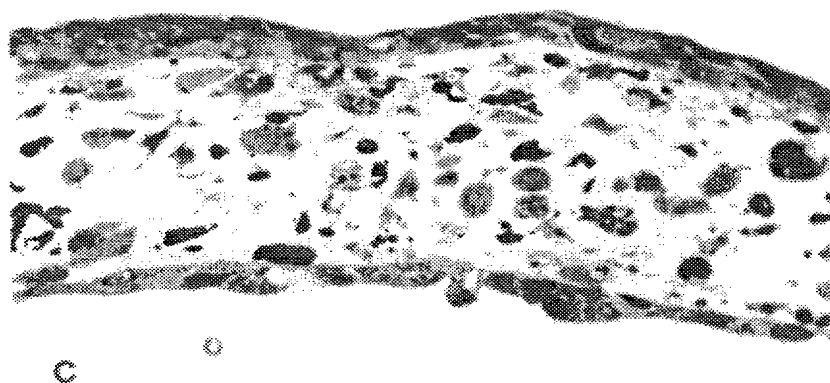


FIG. 3C
SUBSTITUTE SHEET

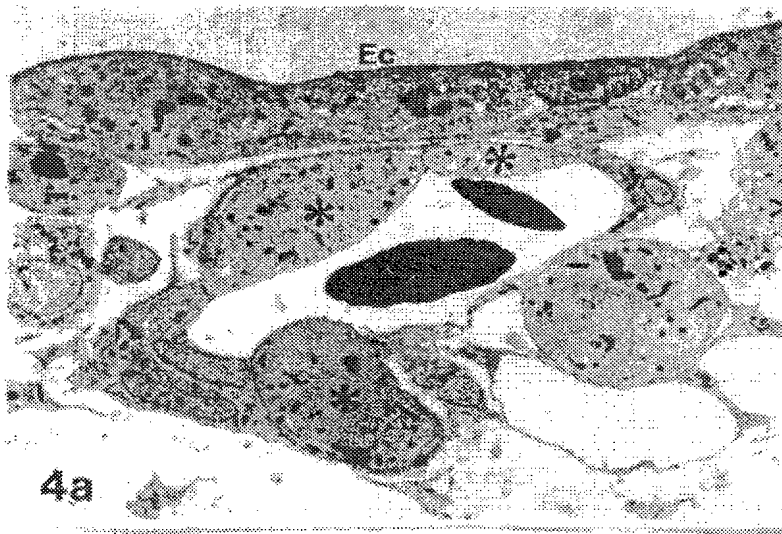


FIG. 4A

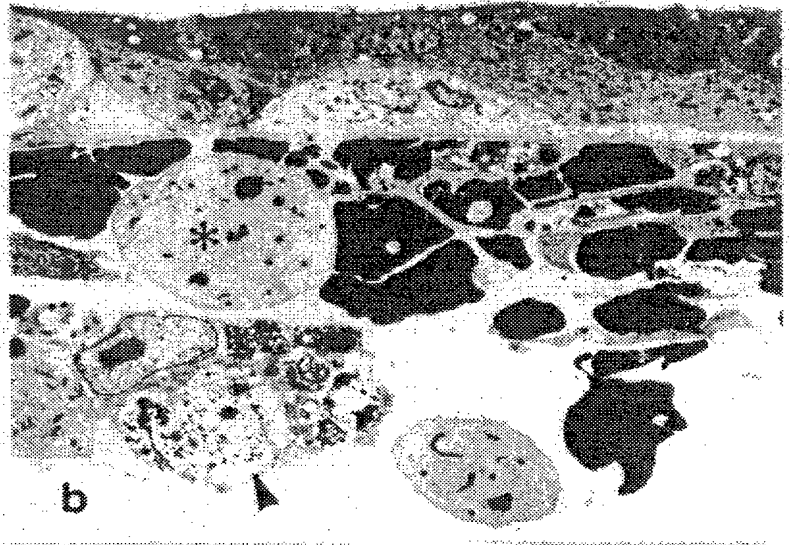


FIG. 4B

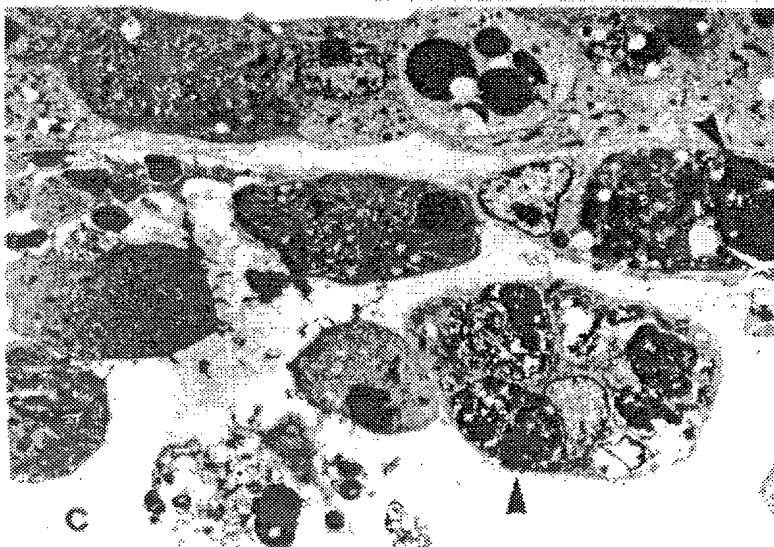


FIG. 4C

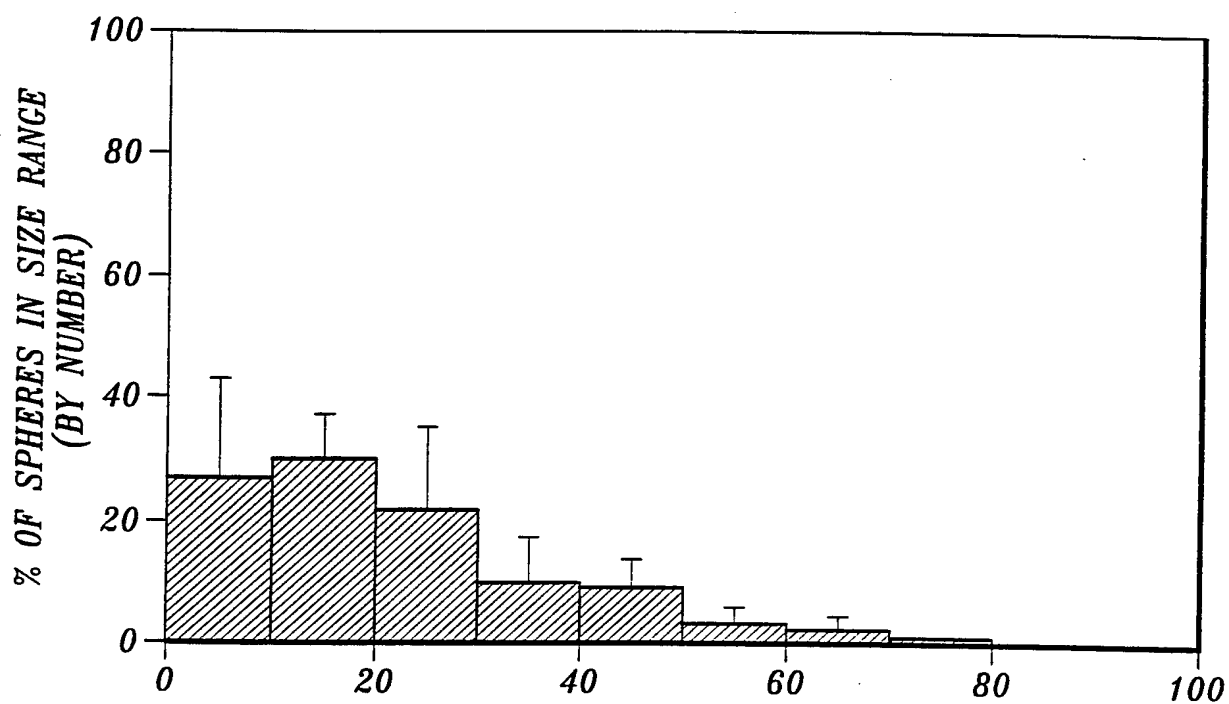


FIG. 5. SIZE RANGE (μ M DIAMETER)

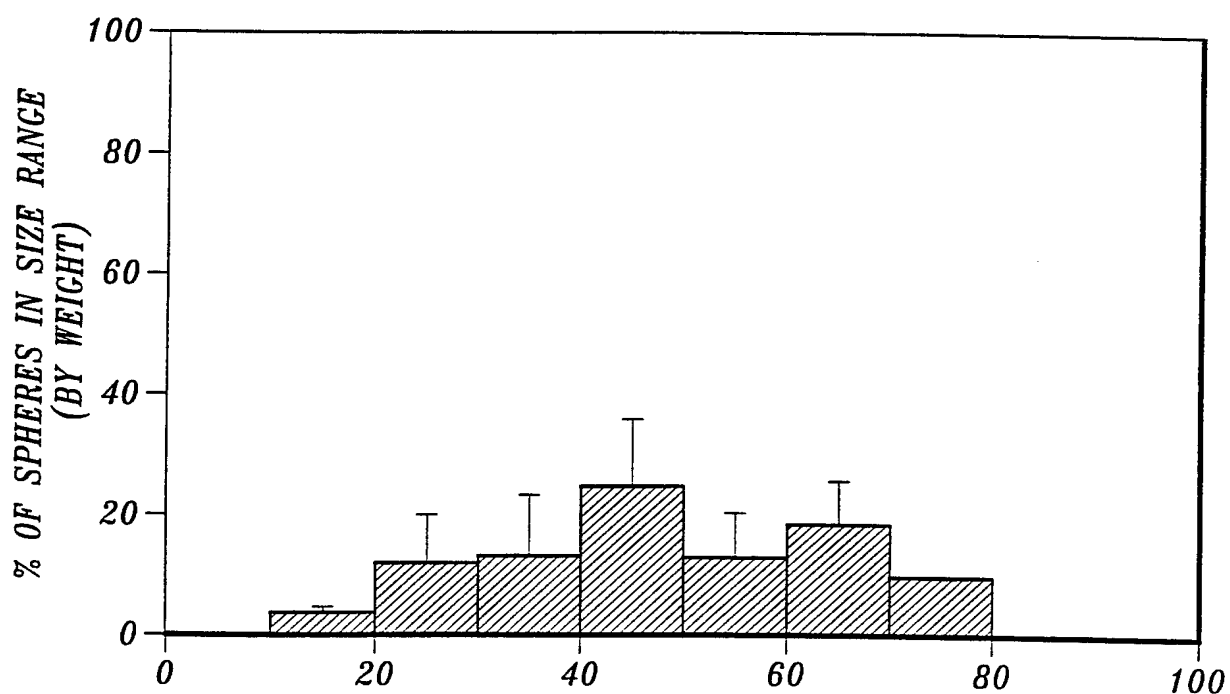


FIG. 6. SIZE RANGE (μ M DIAMETER)

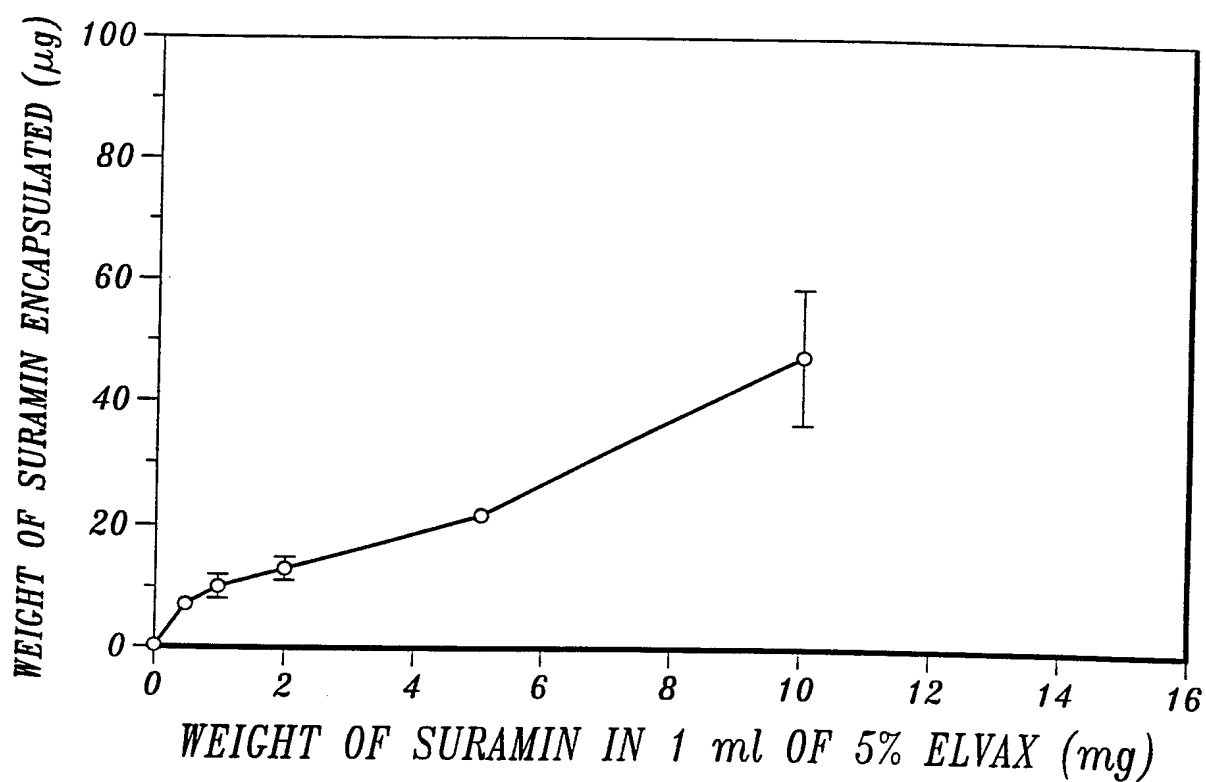


FIG. 7.

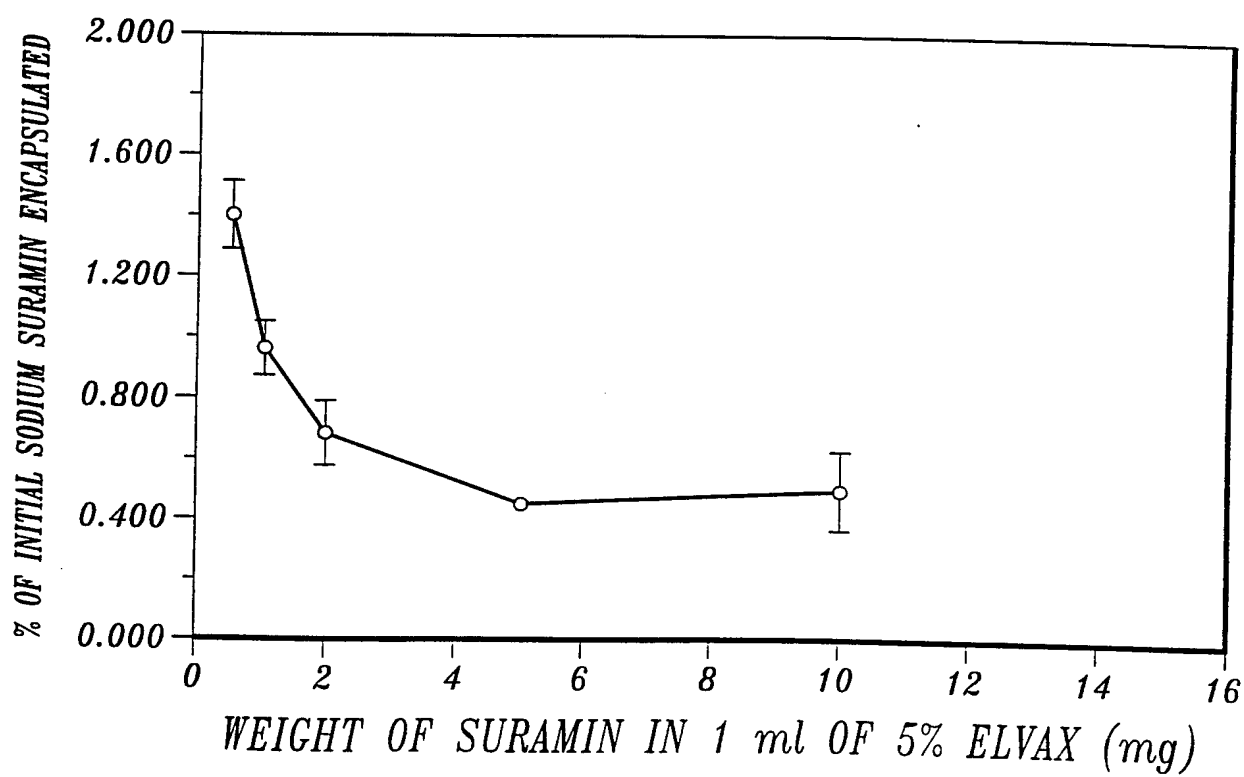


FIG. 8.

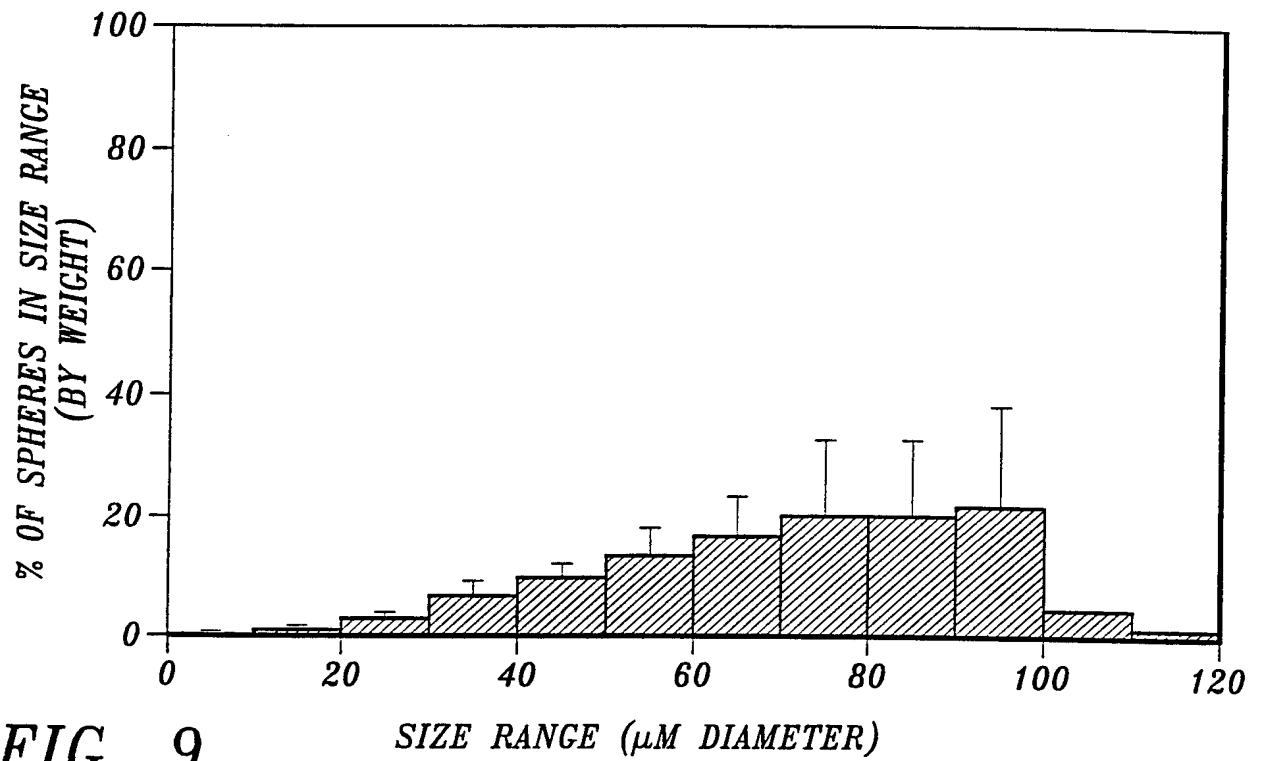


FIG. 9.

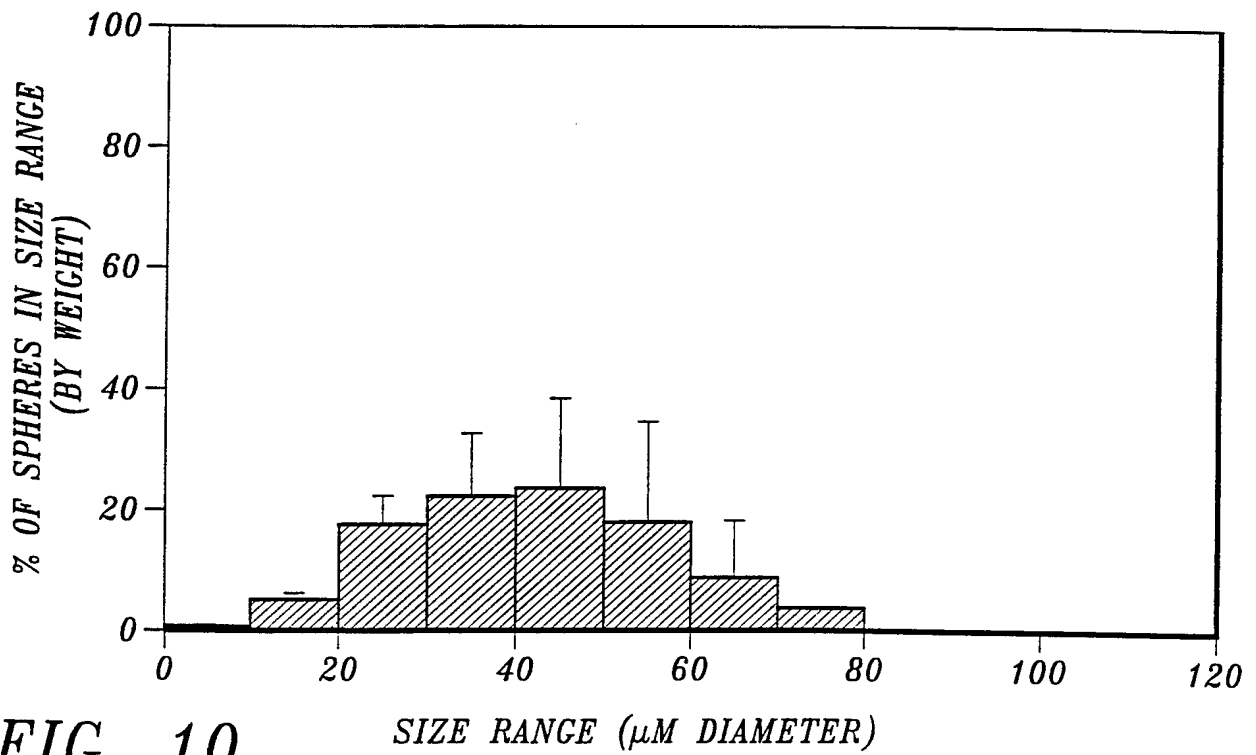


FIG. 10.

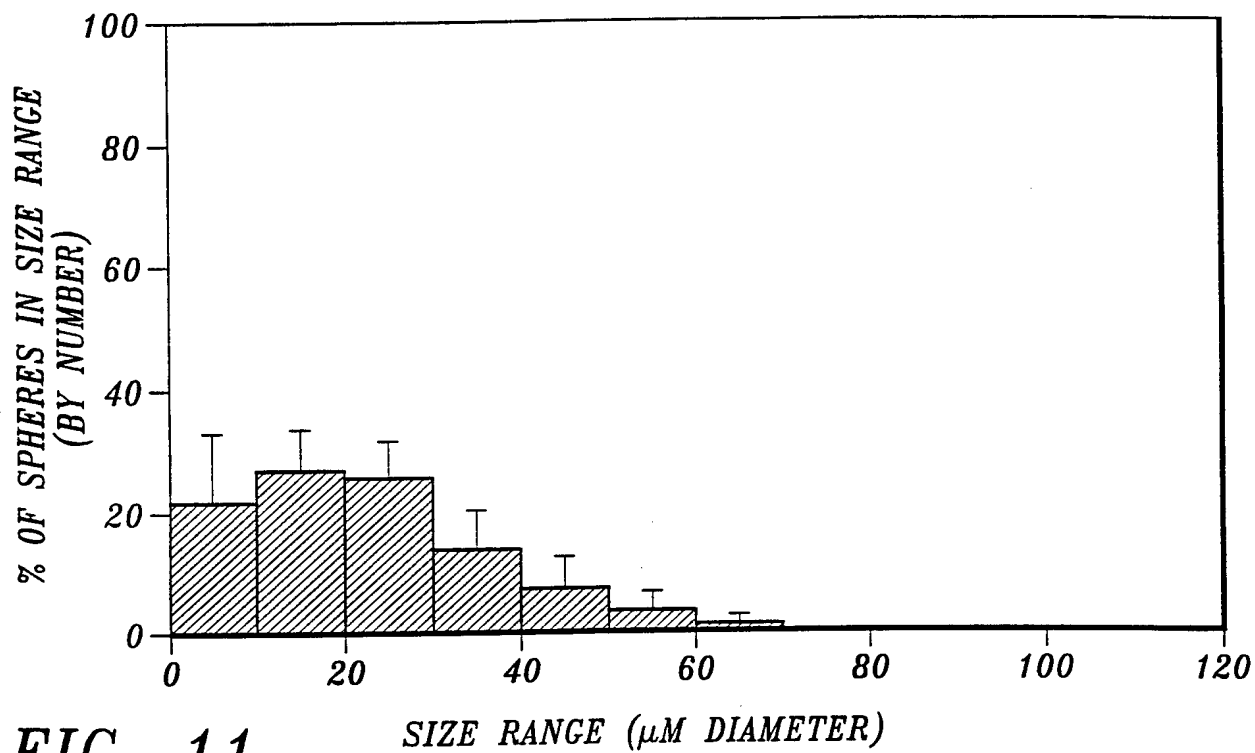


FIG. 11.

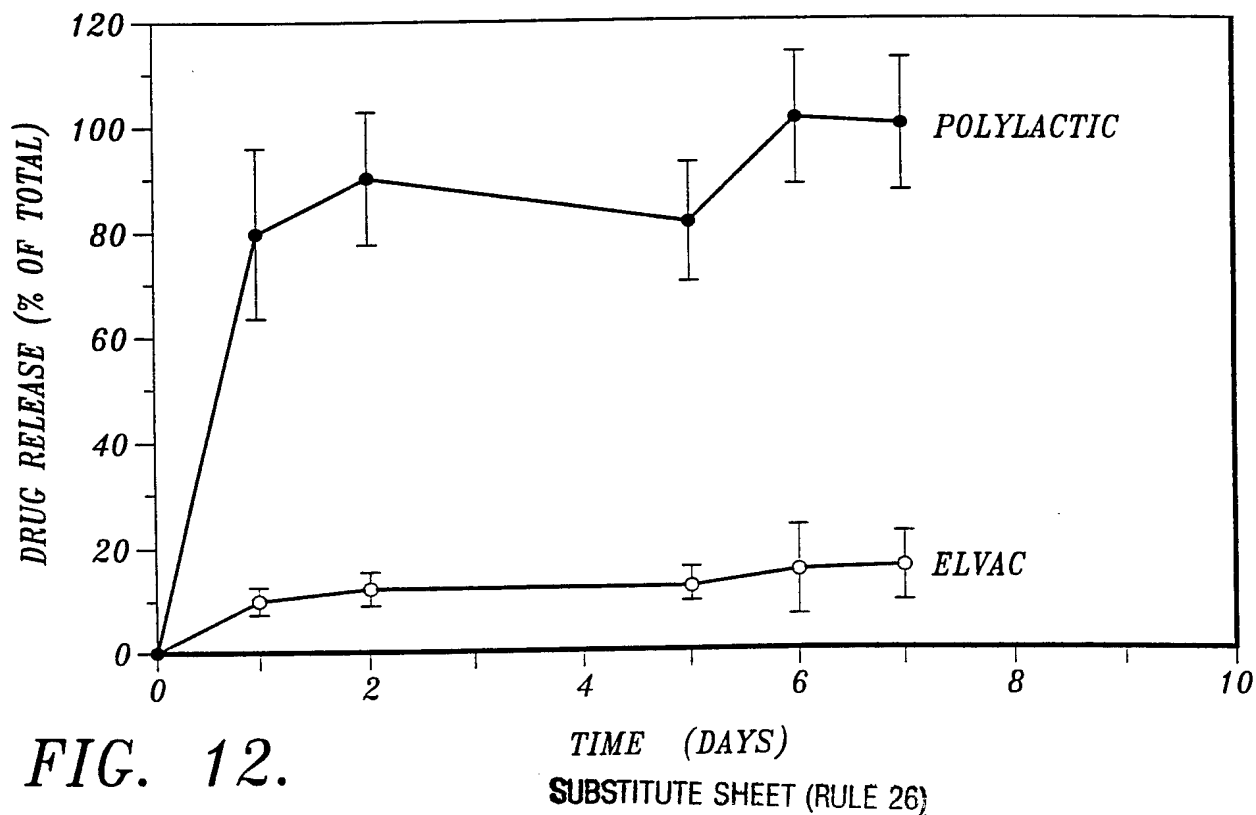
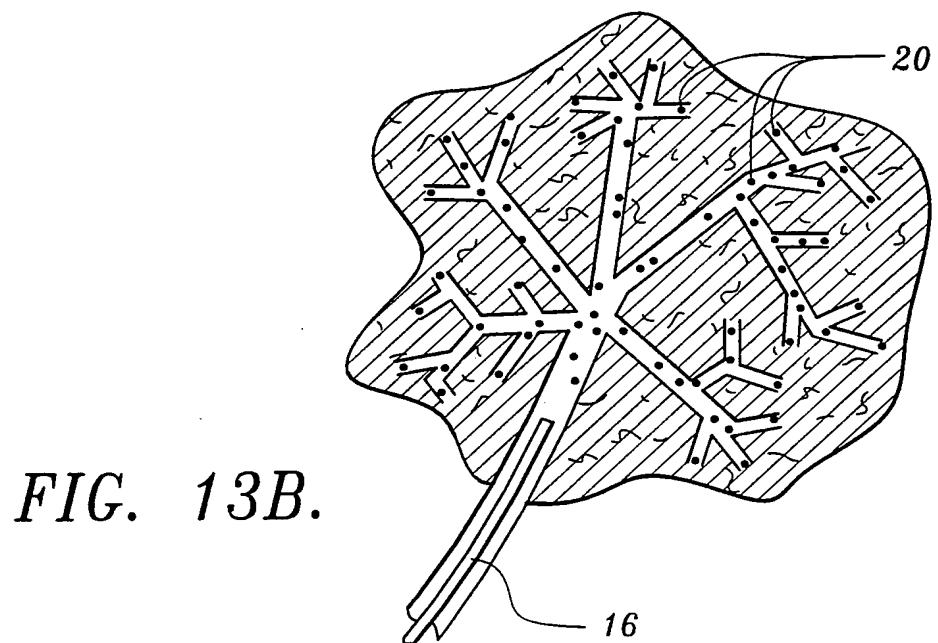
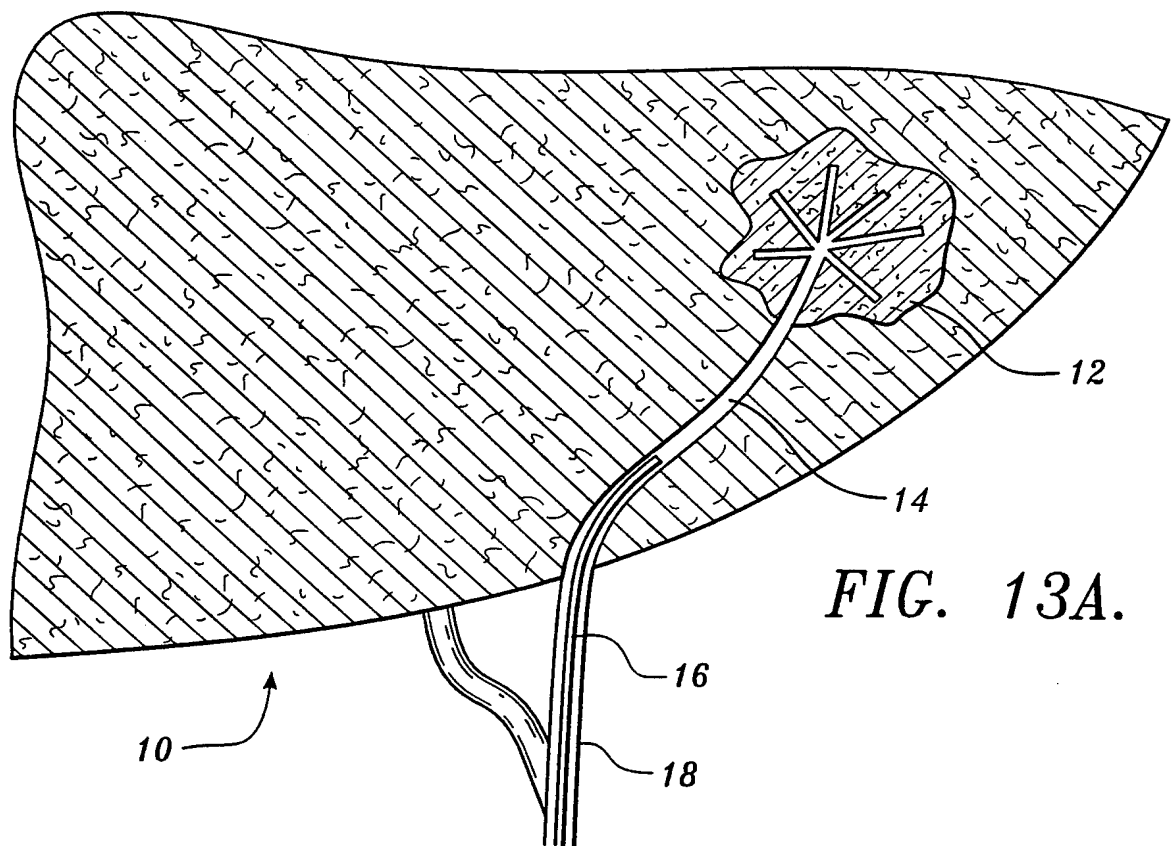
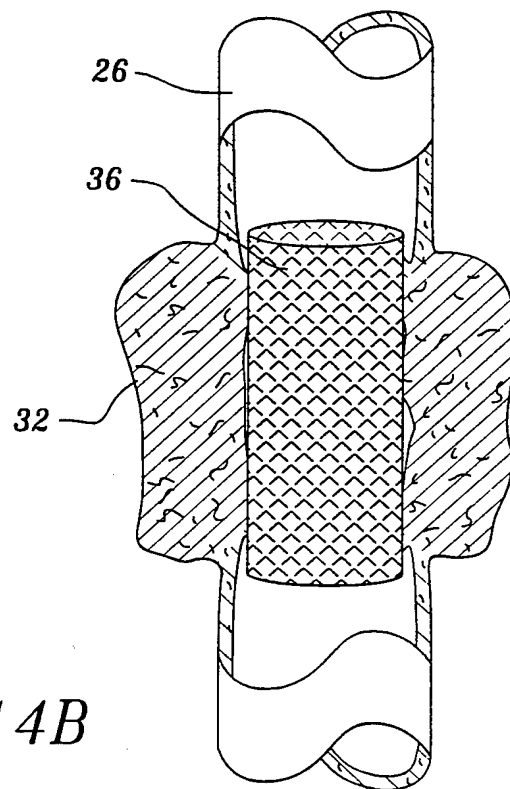
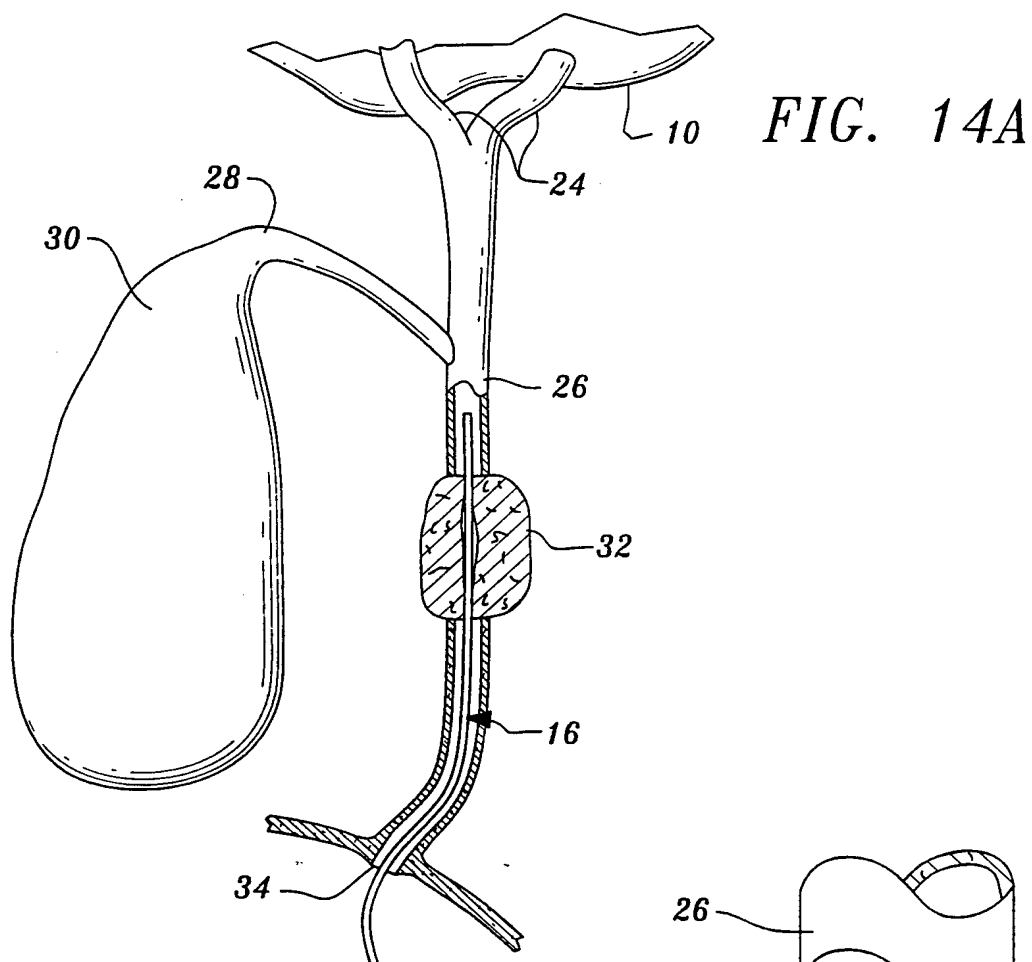
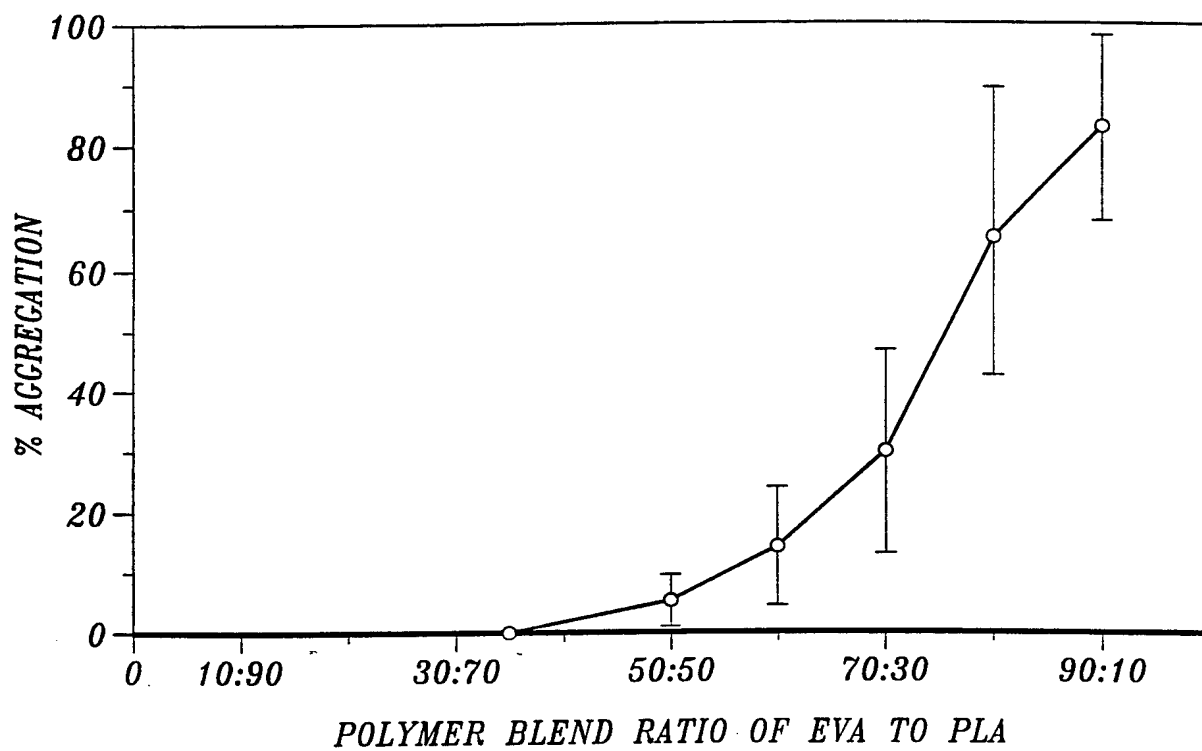


FIG. 12.



12/36



*FIG. 15A.*

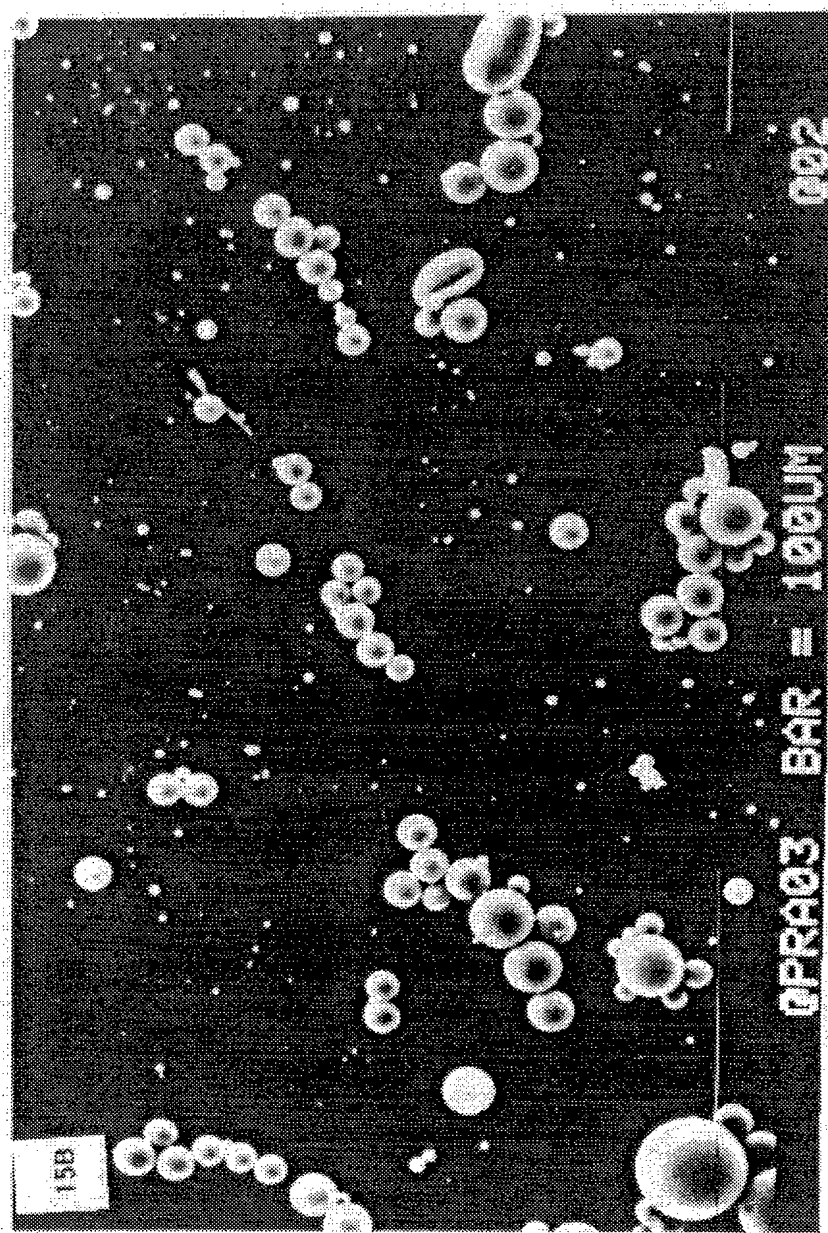


FIG. 15B

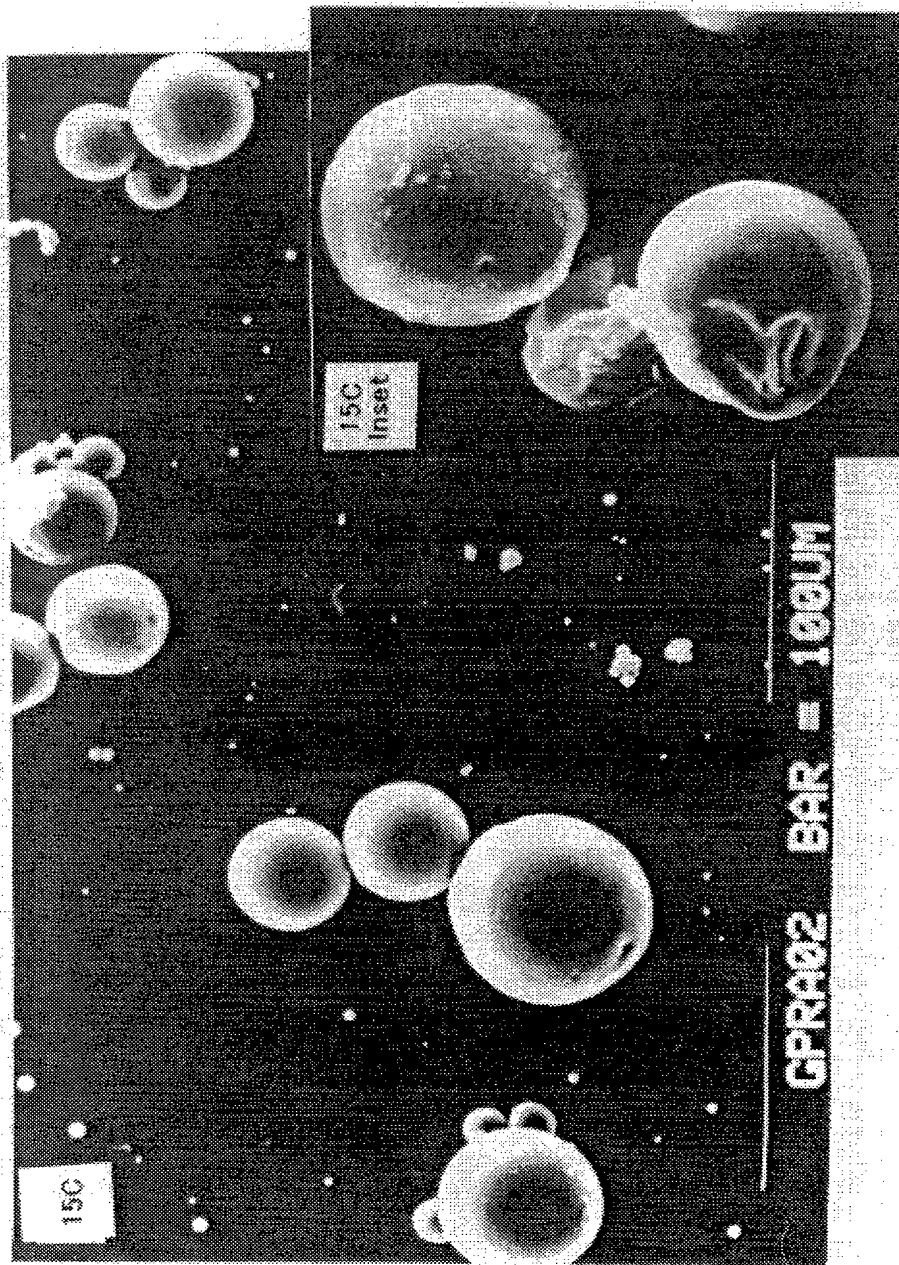
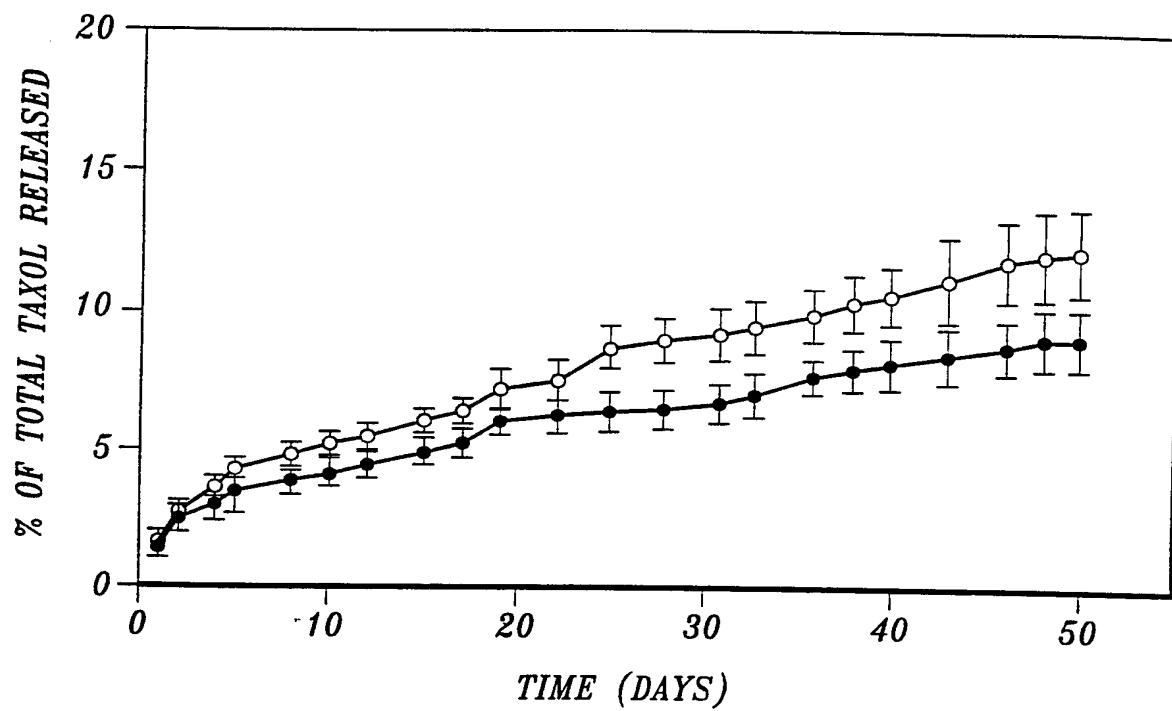


FIG. 15C

*FIG. 15D.*

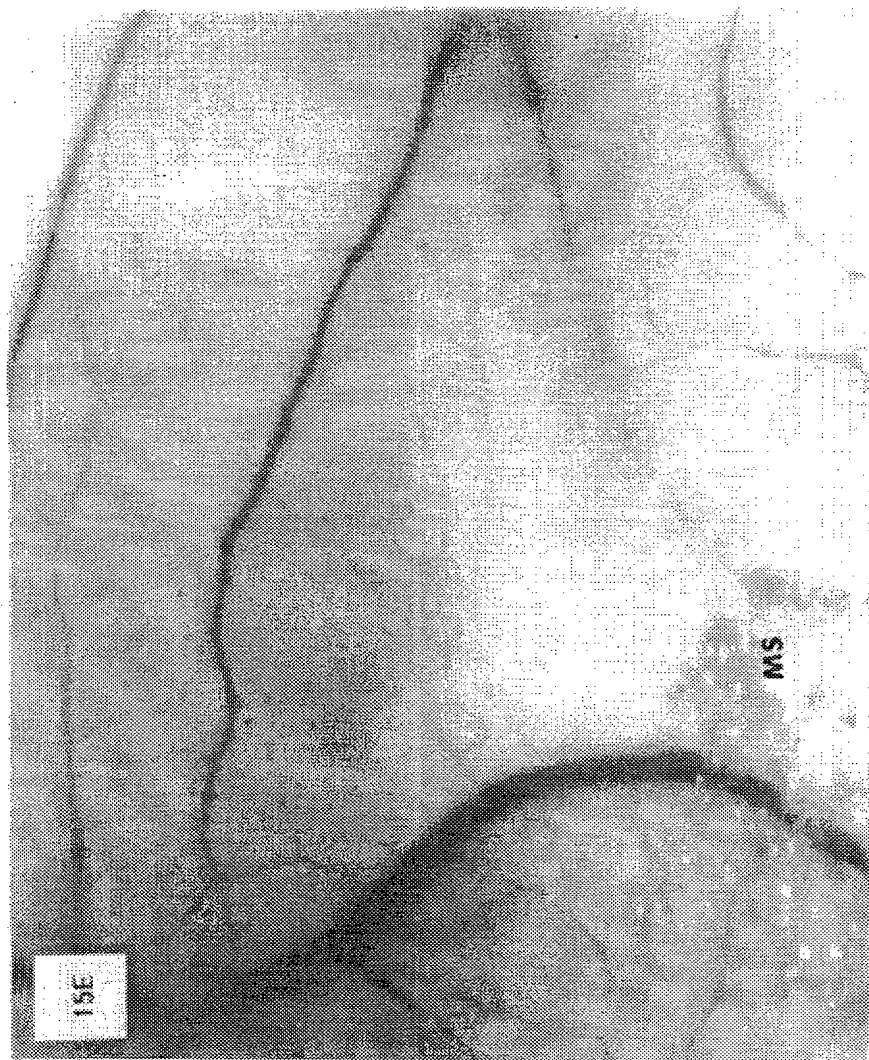


FIG. 15E

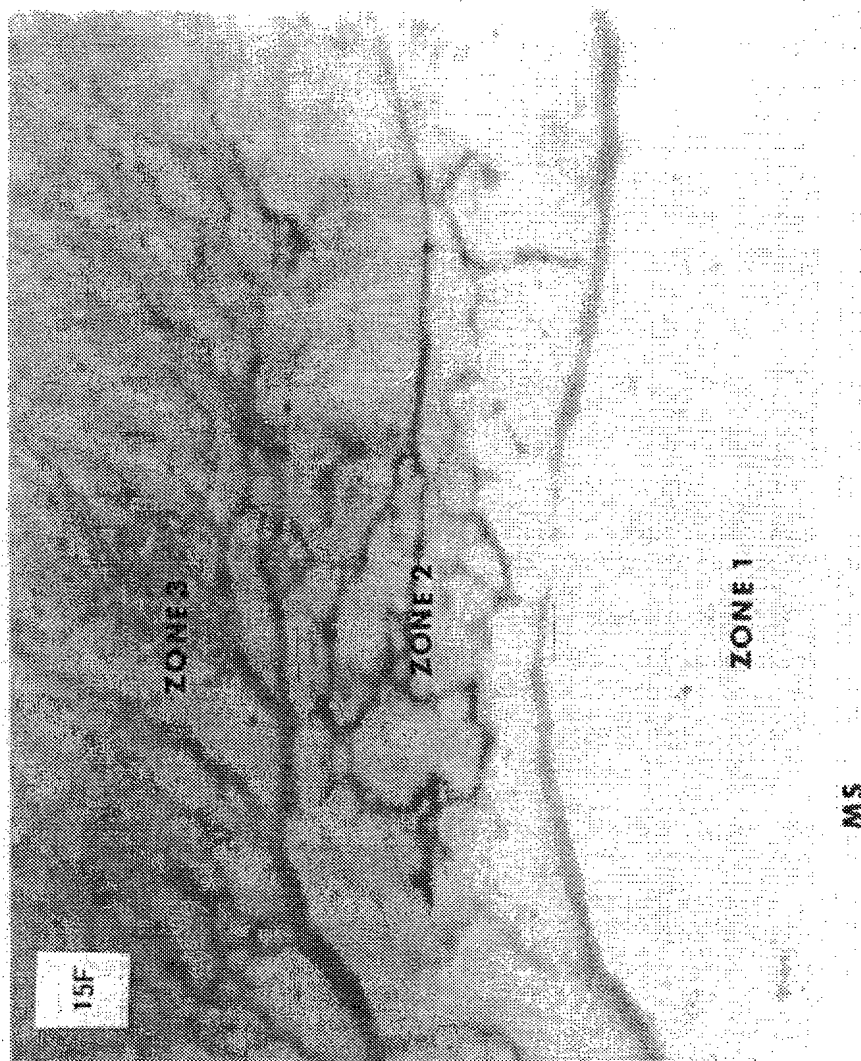


FIG. 15F

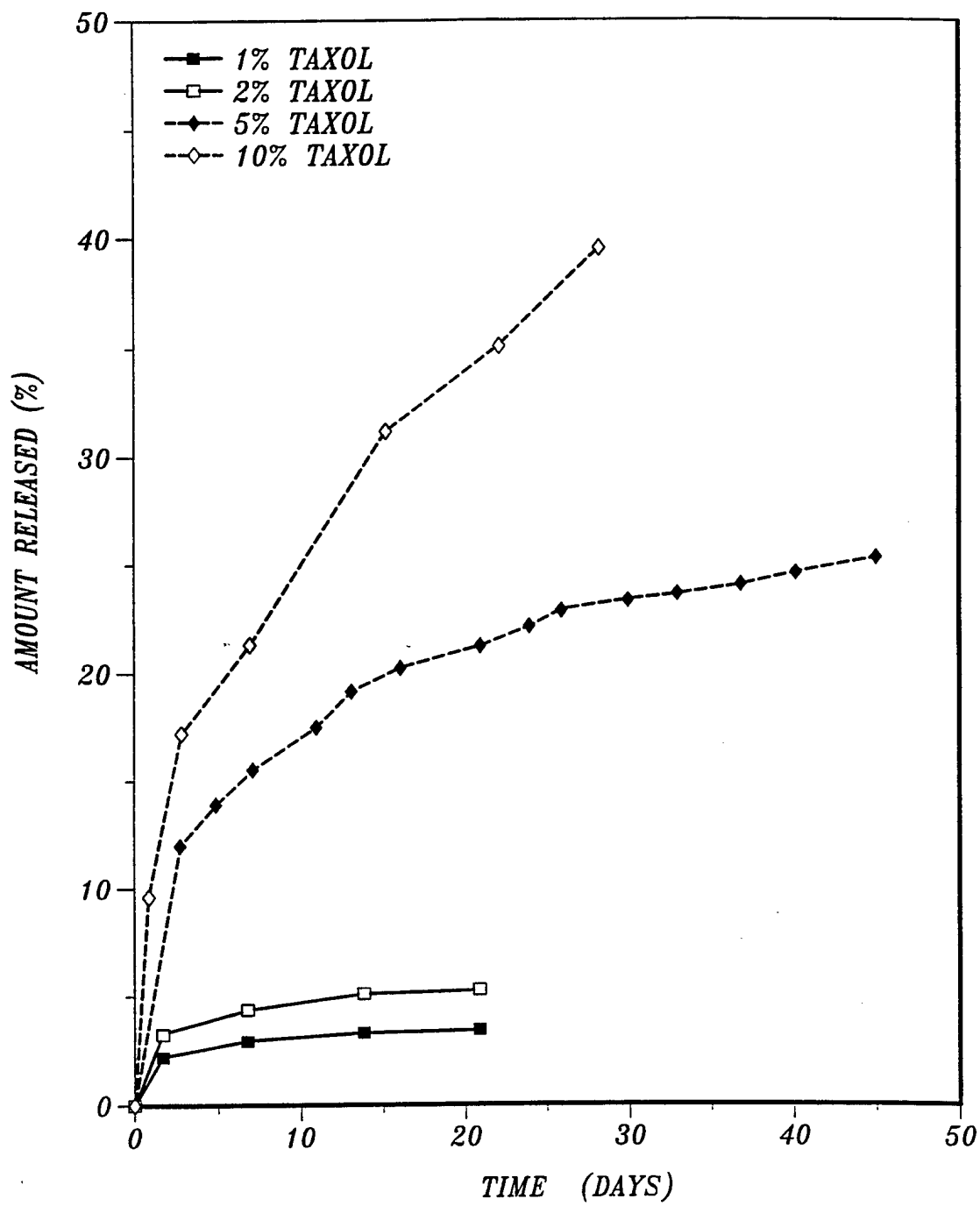


FIG. 16A.

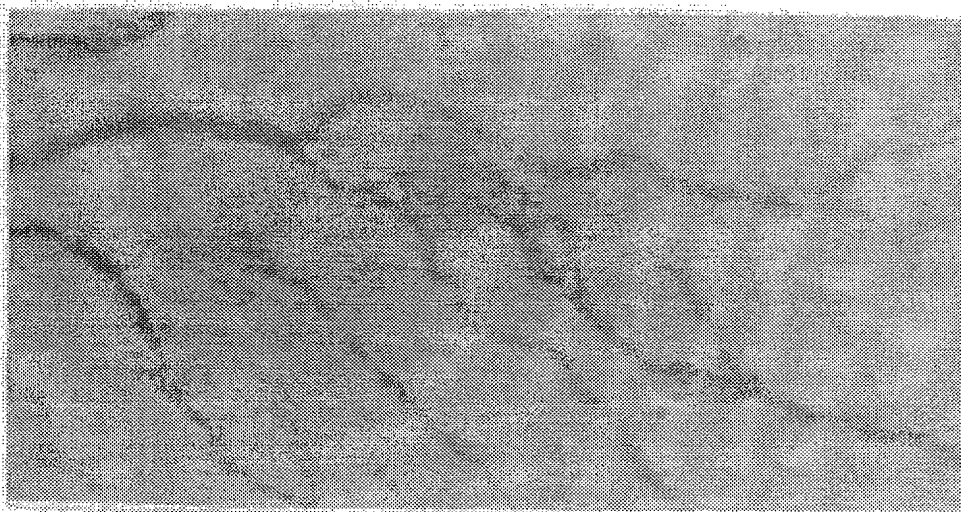


FIG. 16B

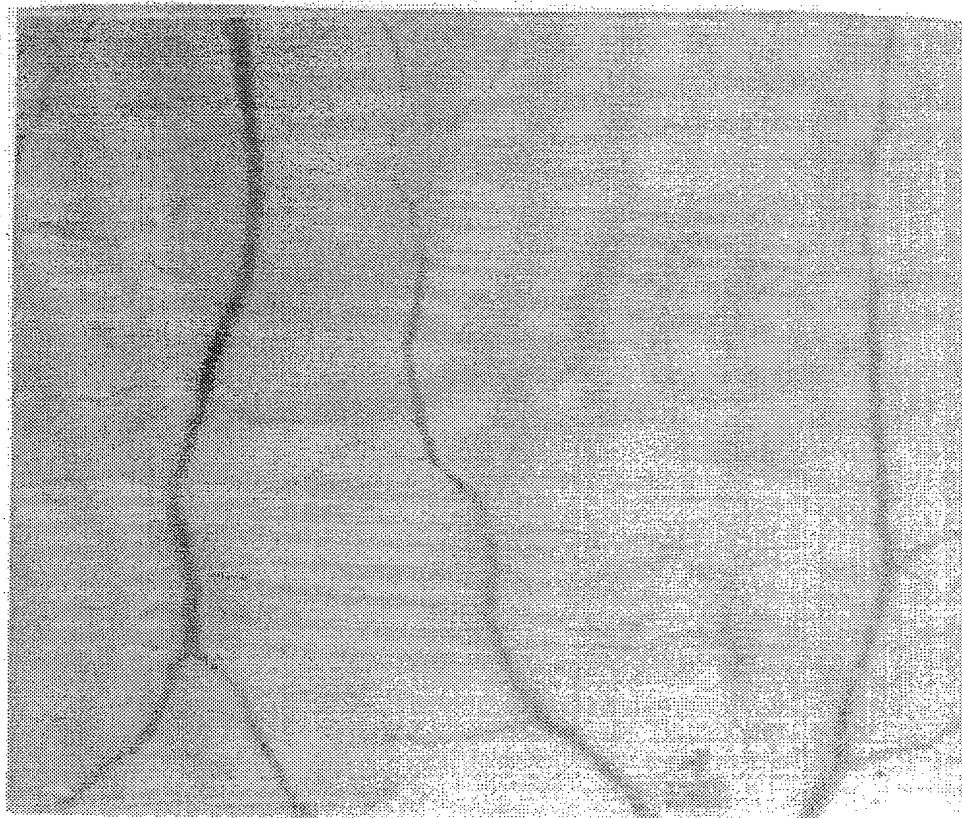


FIG. 16C

SUBSTITUTE SHEET

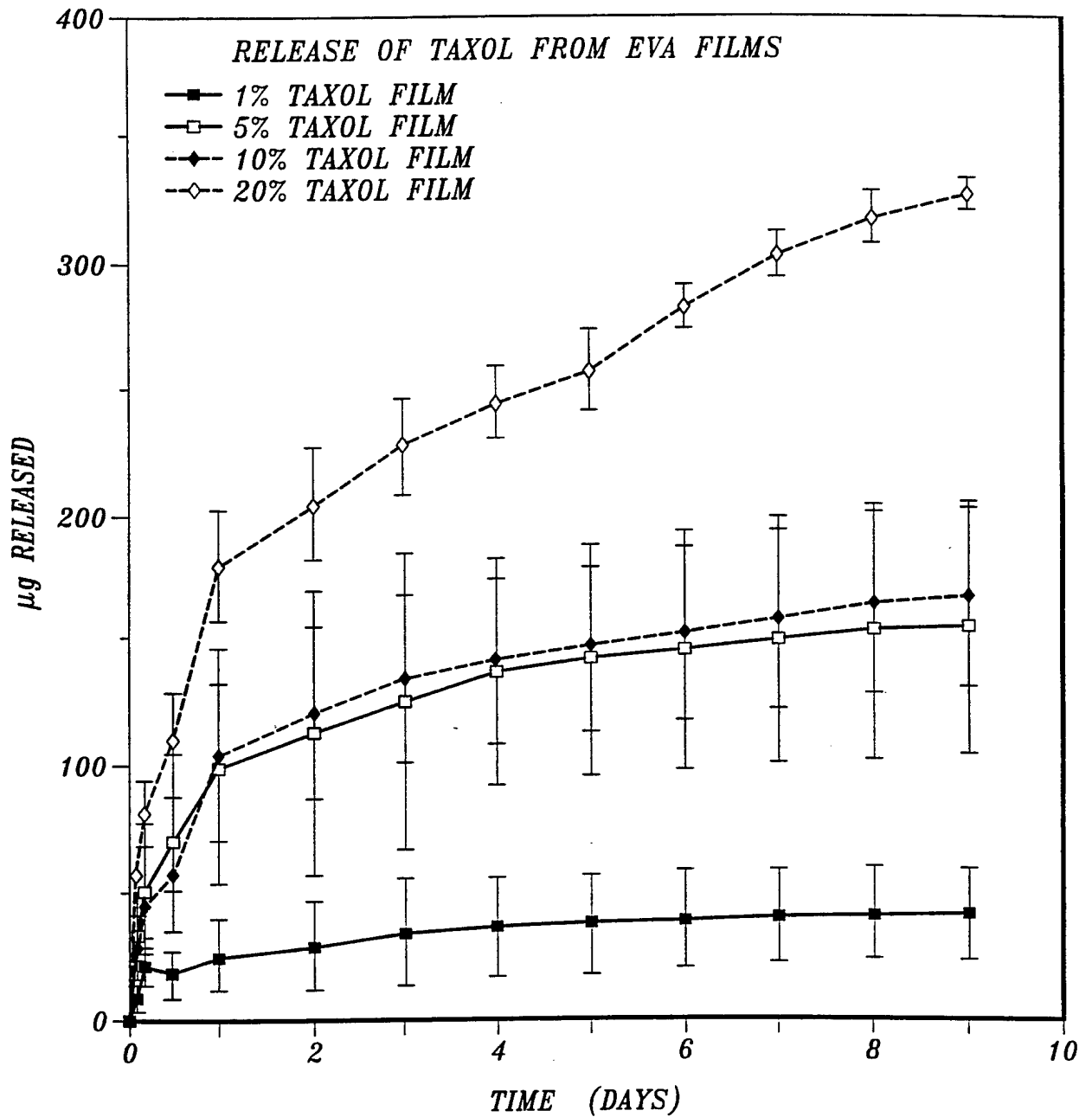
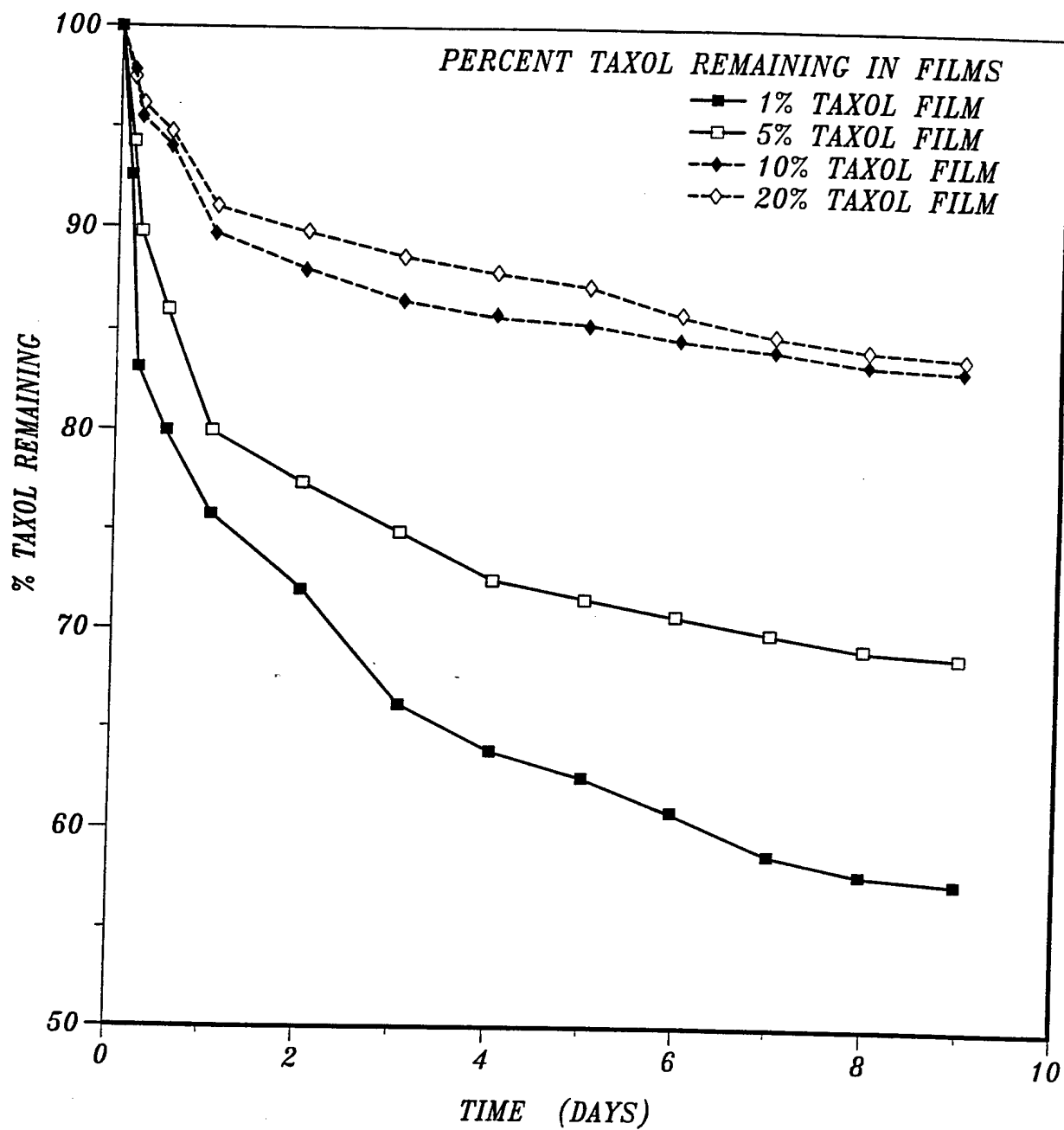


FIG. 17A.

*FIG. 17B.*

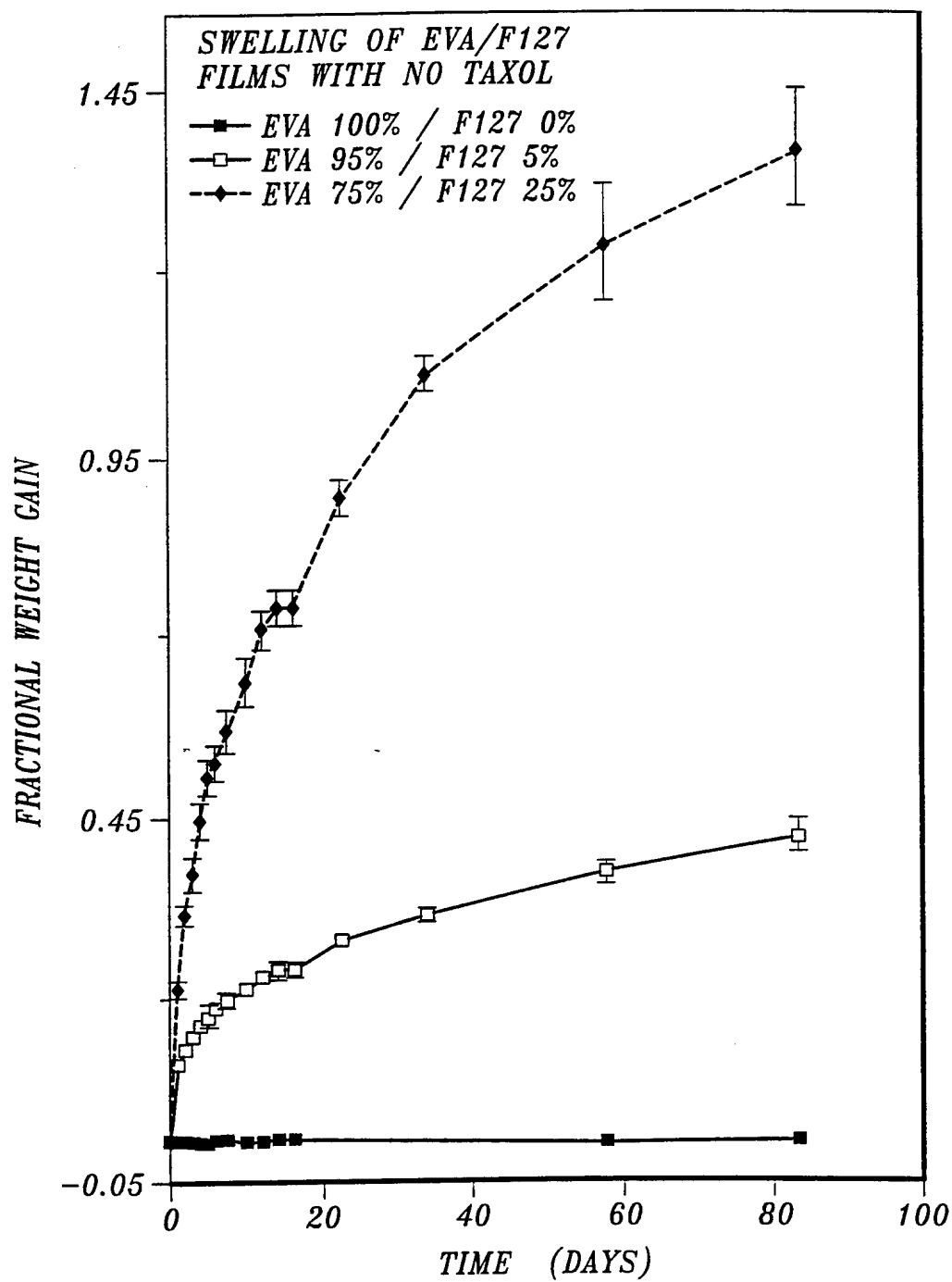
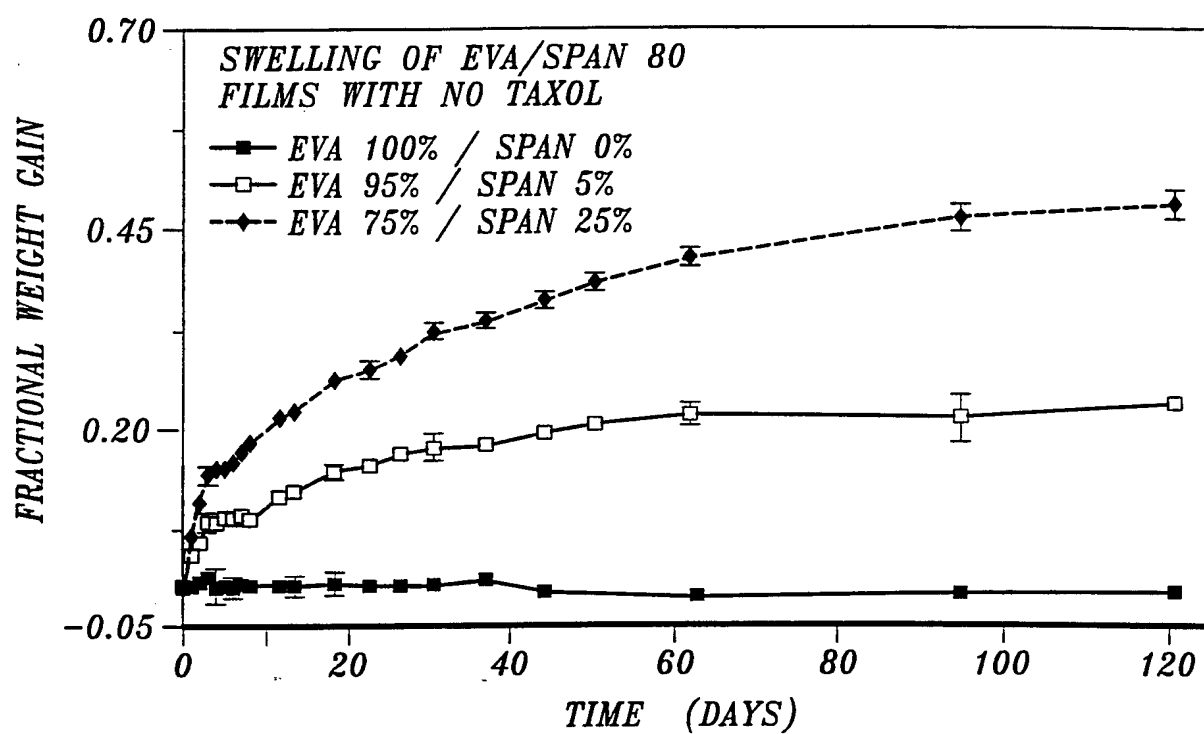


FIG. 17C.

*FIG. 17D.*

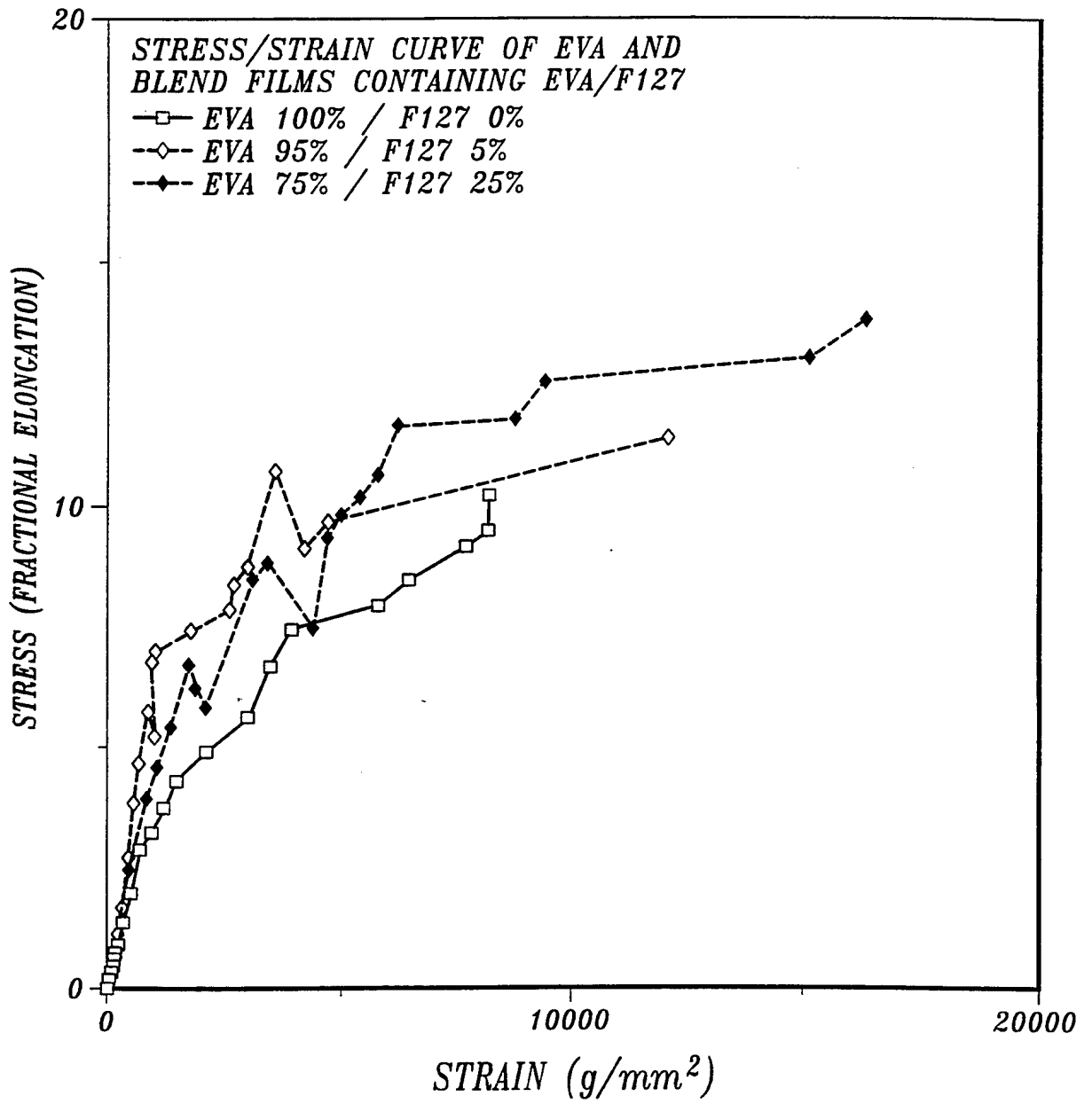
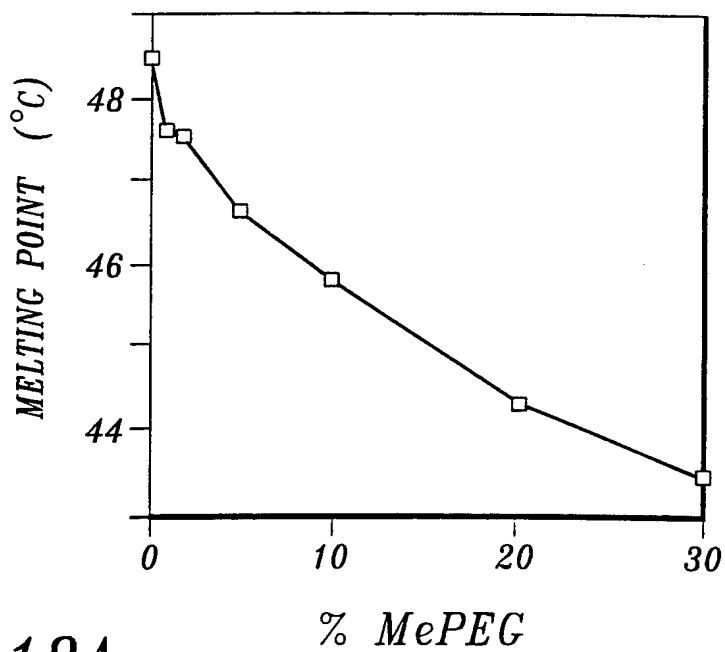
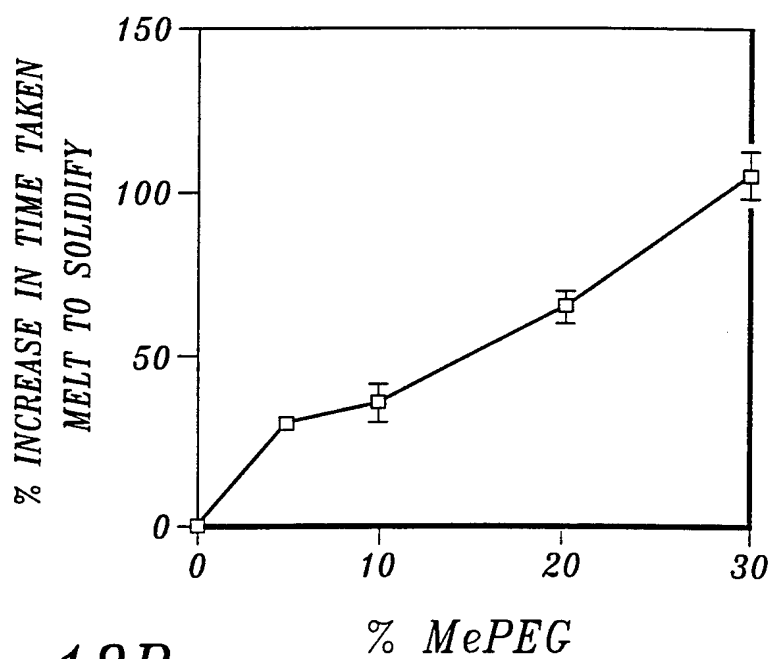


FIG. 17E.

*FIG. 18A.**FIG. 18B.*

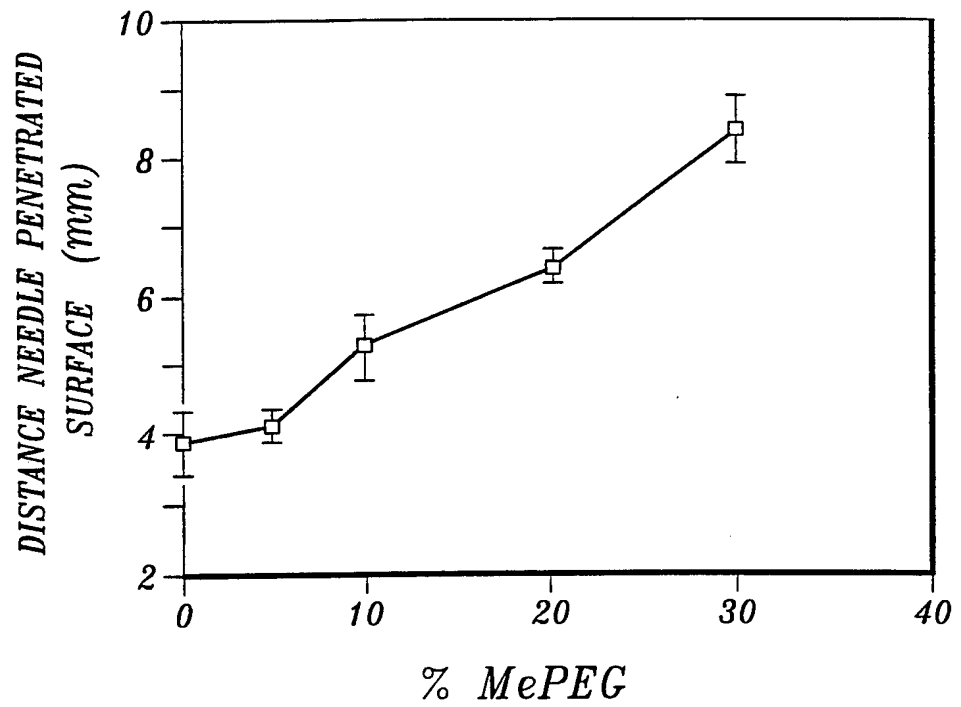


FIG. 18C.

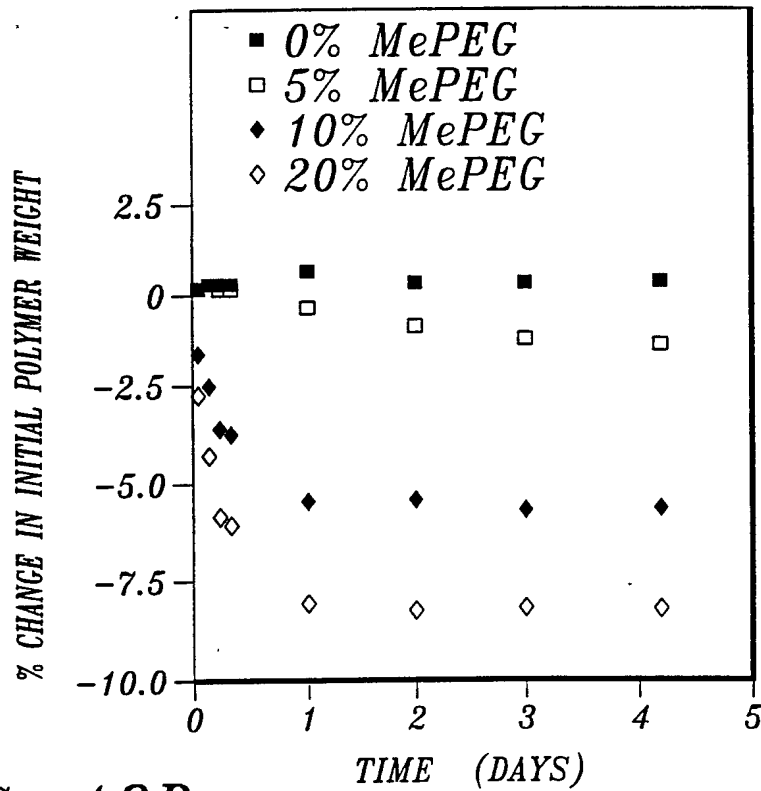
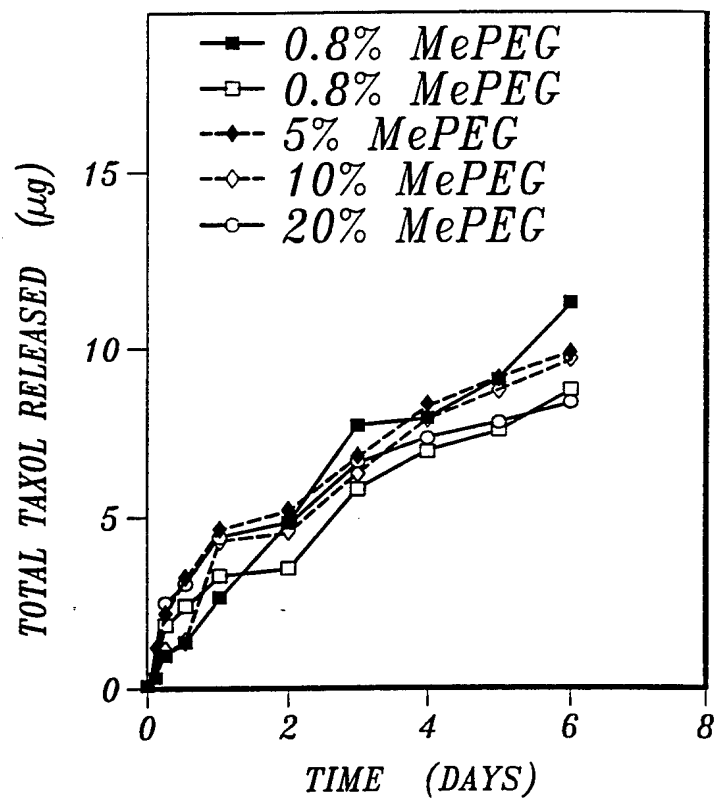


FIG. 18D.

*FIG. 18E.*

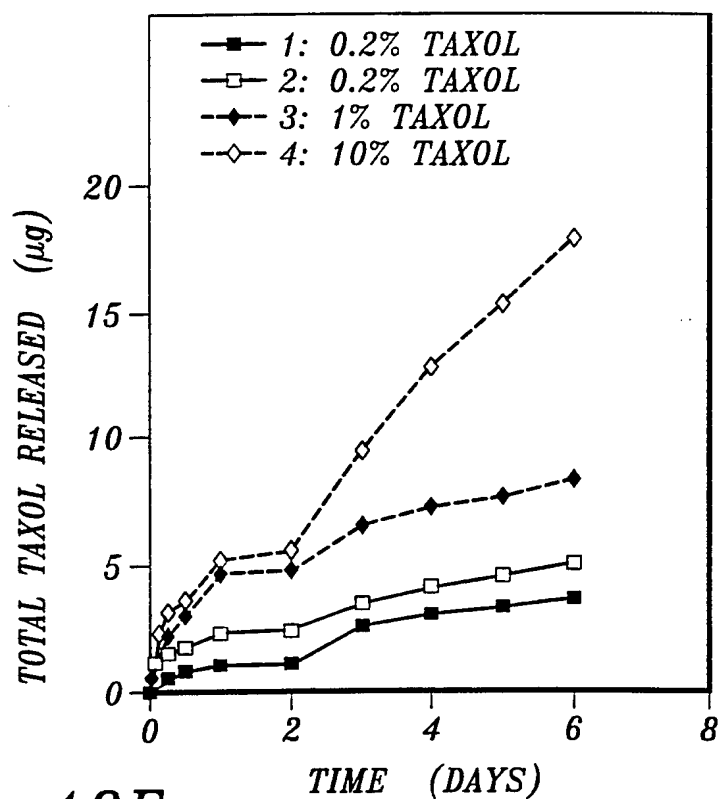


FIG. 18F.

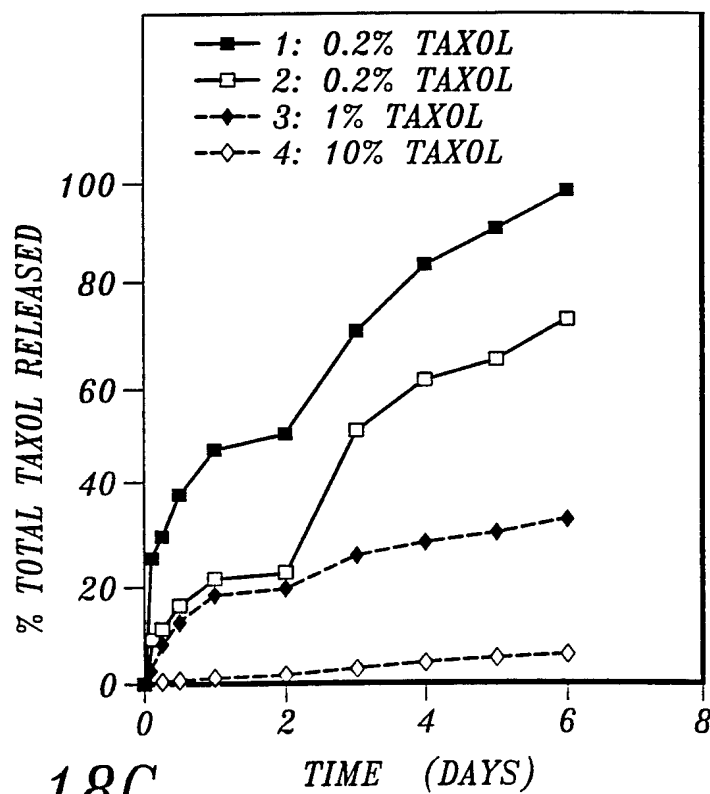


FIG. 18G.

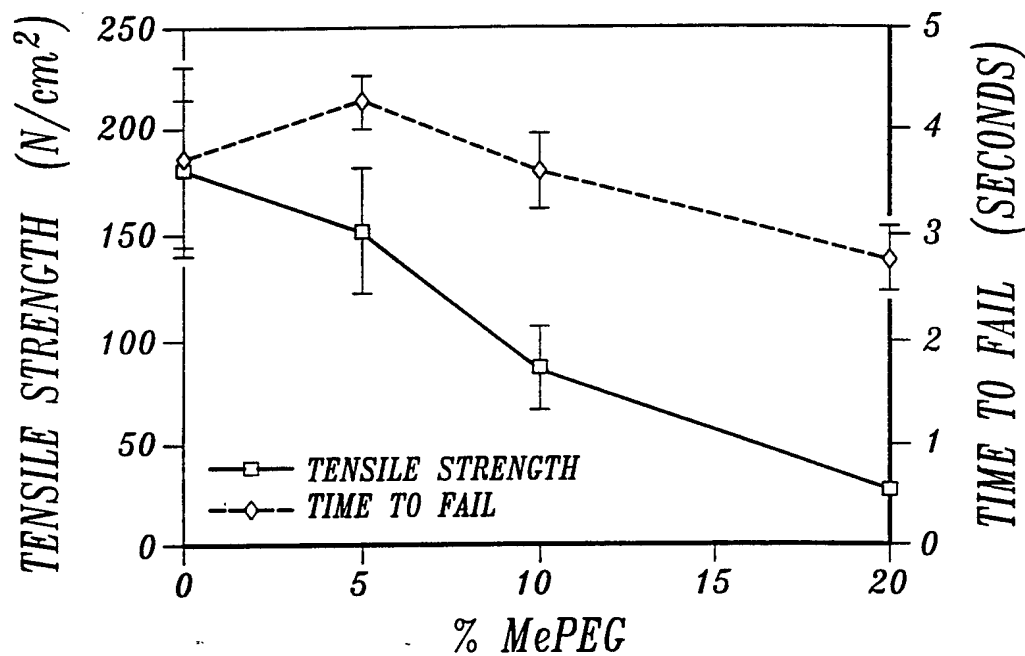
*FIG. 18H.*



FIG. 19A

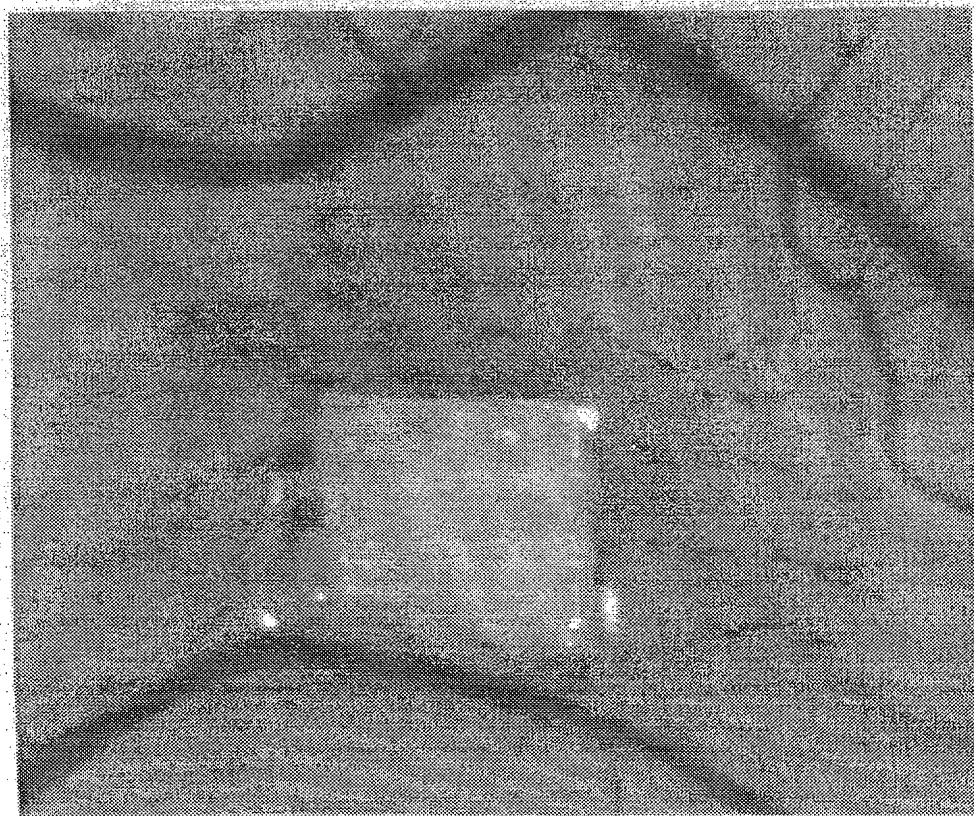


FIG. 19B

SUBSTITUTE SHEET

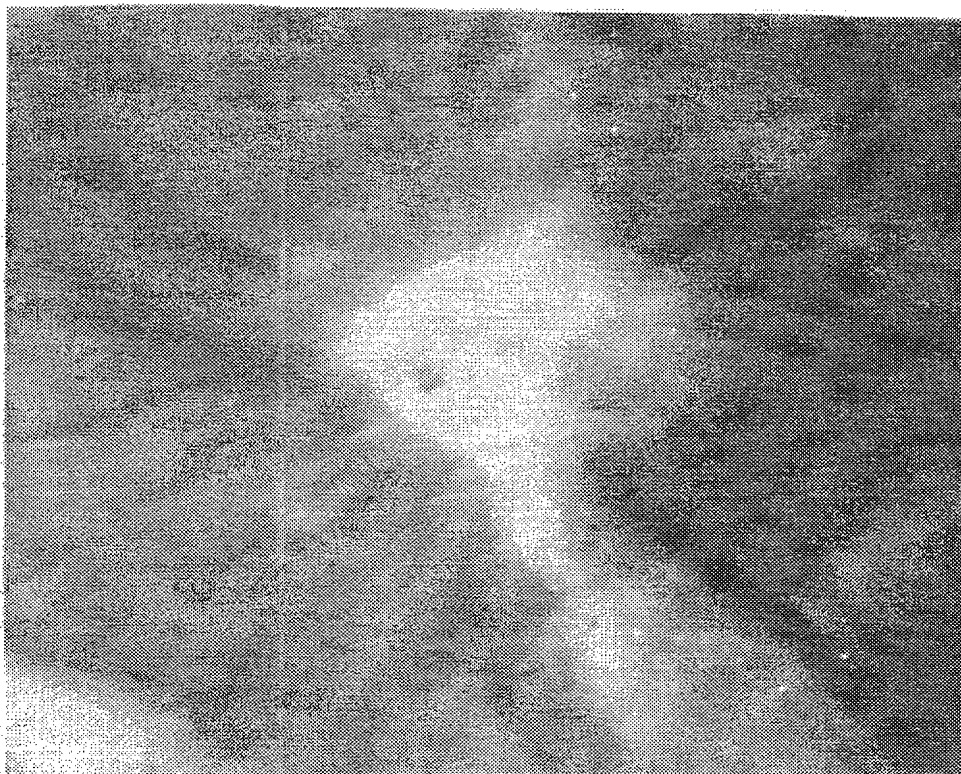


FIG. 20A

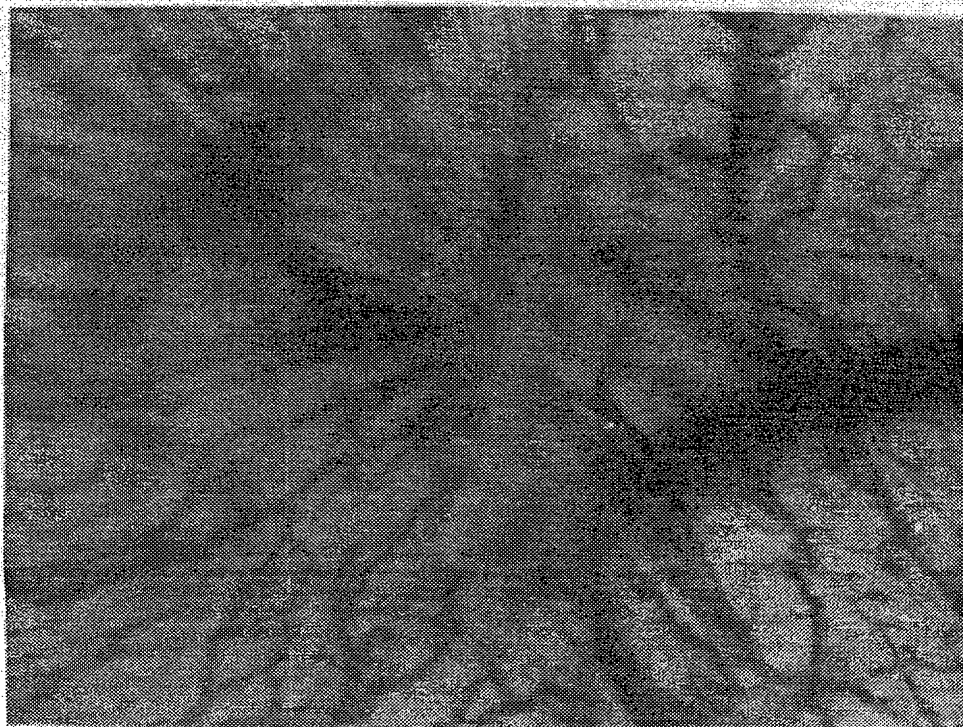


FIG. 20B
SUBSTITUTE SHEET



FIG. 20C

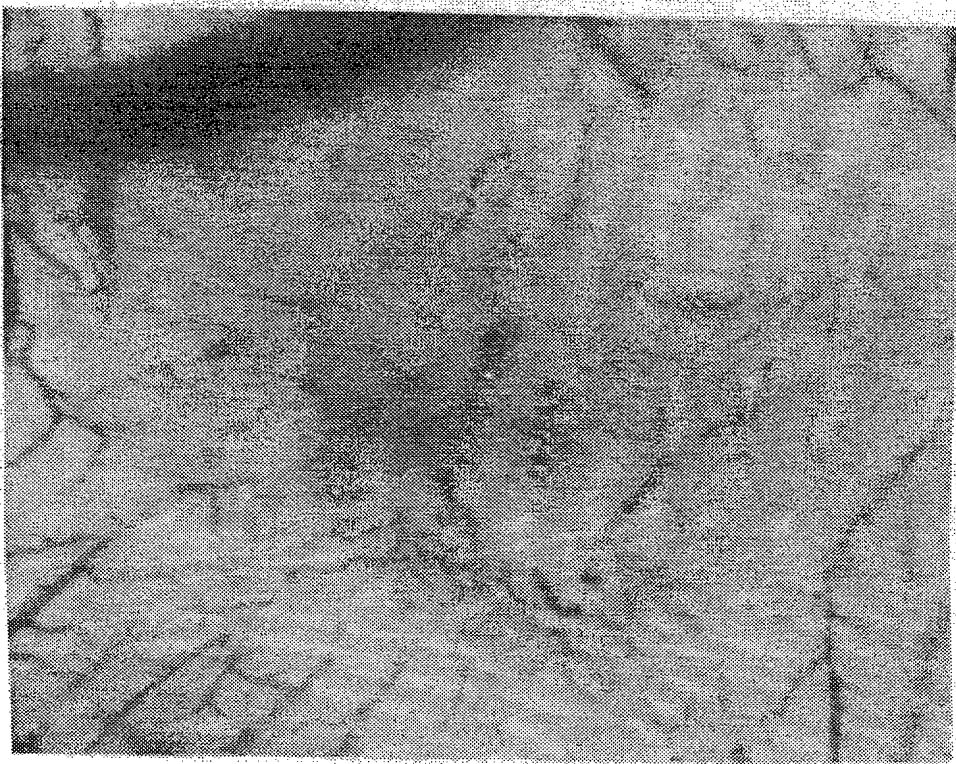
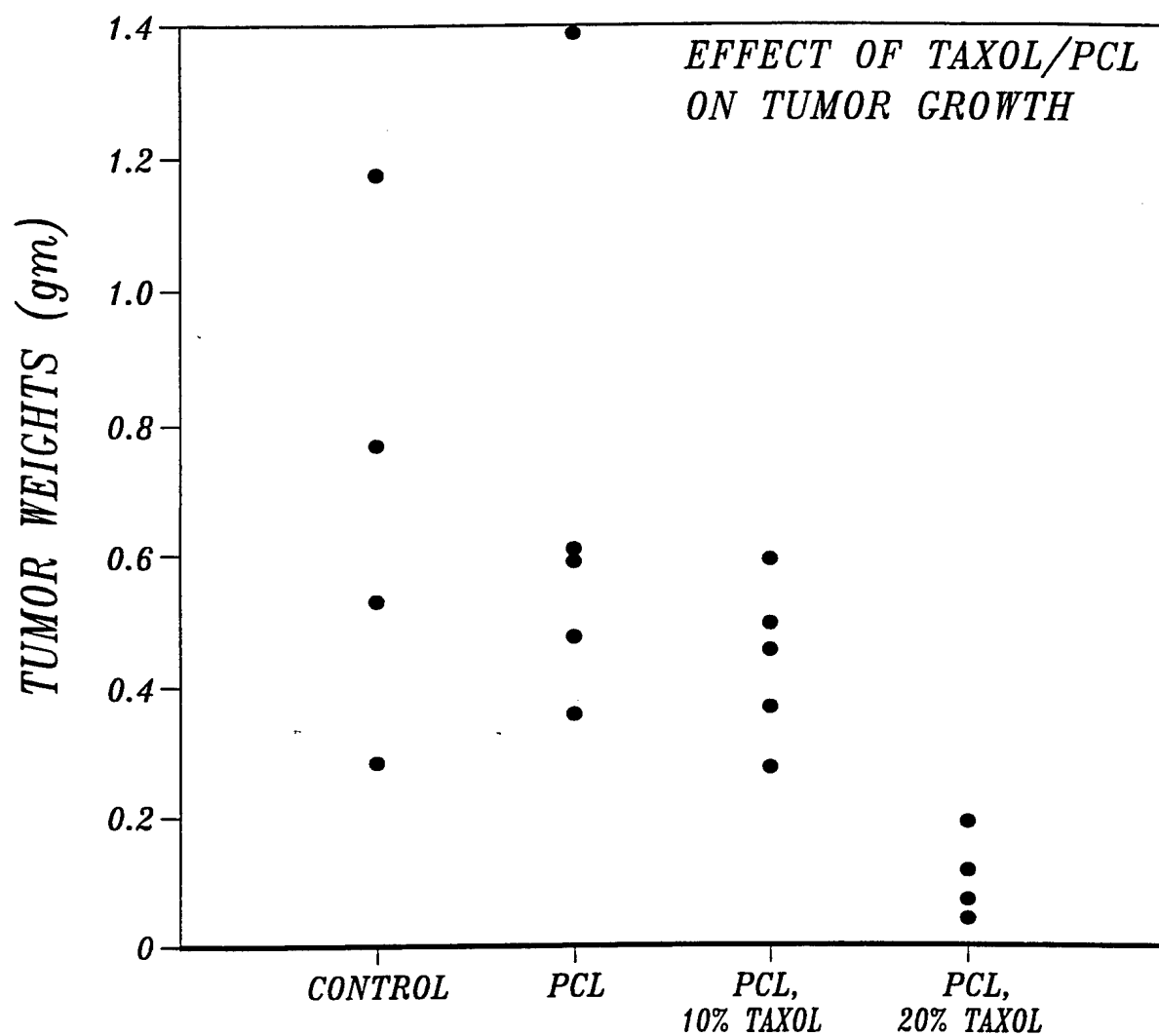
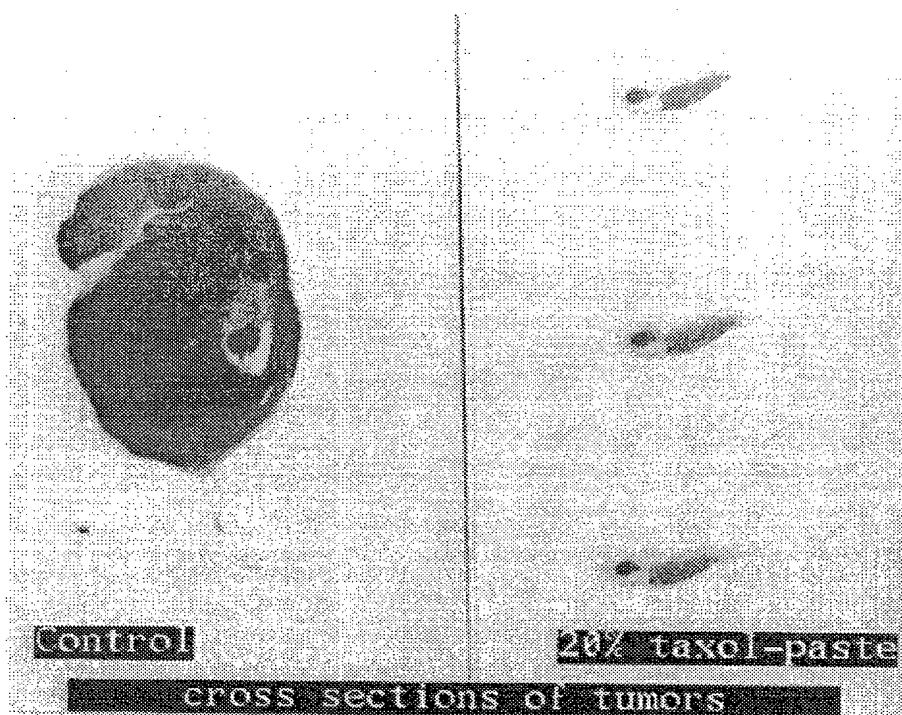
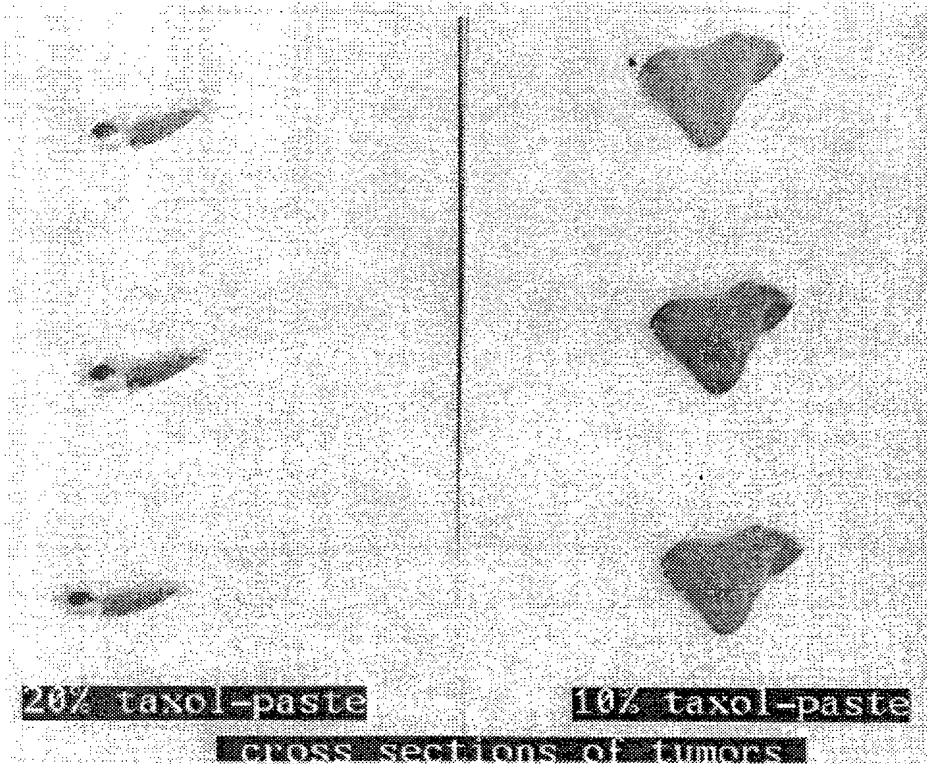


FIG. 20D

SUBSTITUTE SHEET

*FIG. 21A.*

*FIG. 21B**FIG. 21C*

SUBSTITUTE SHEET

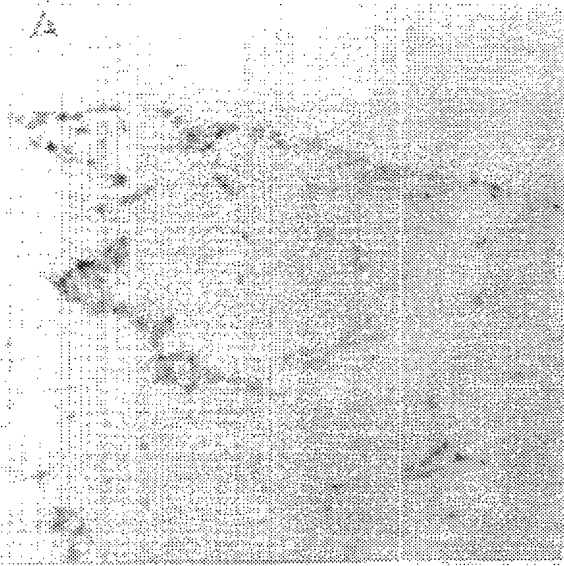


FIG. 22A

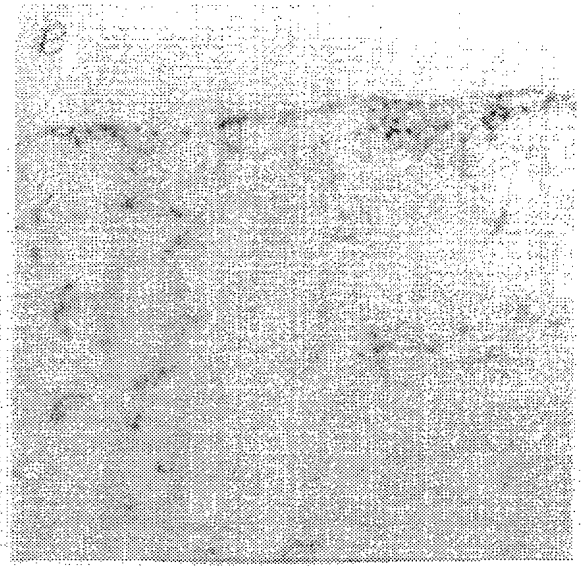


FIG. 22B

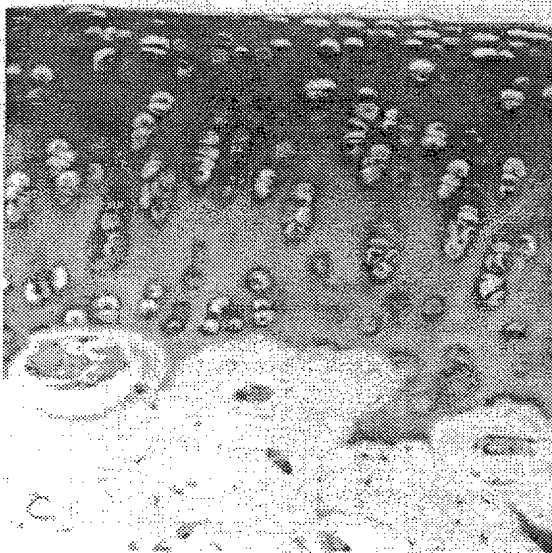


FIG. 22C

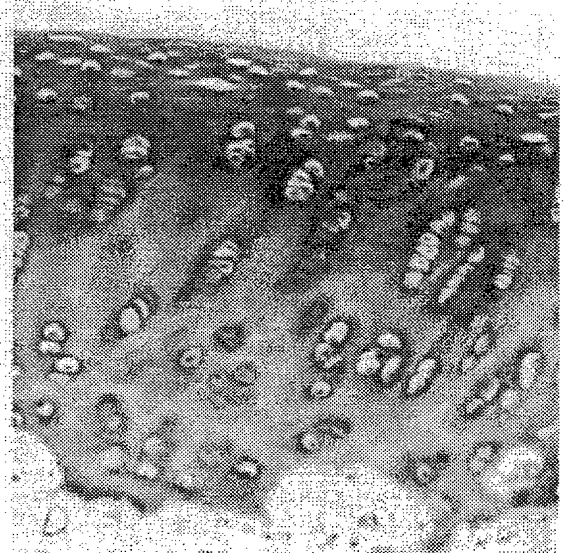


FIG. 22D

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/CA 94/00373

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K9/16 A61K9/70 A61L31/00 A61K31/20 A61K31/335
A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 470 569 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 12 February 1992 see the whole document ---	1,6,8, 10-16
X	WO,A,91 11193 (CHILDREN'S HOSPITAL) 8 August 1991	1,2,4
Y	see page 1, line 8 - page 6, line 21 see page 27, line 27 - line 34 see claims 11,23 ---	17-23, 31-35
X	WO,A,91 10424 (NORTHWESTERN UNIVERSITY) 25 July 1991 see page 1, line 1 - line 3 see page 13, line 12 - line 23 see page 19 - page 20; examples 7,8 --- -/--	1,6,9,24

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

22 December 1994

Date of mailing of the international search report

30.12.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Benz, K

INTERNATIONAL SEARCH REPORT

L International Application No
PCT/CA 94/00373

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 375 520 (CENTRE INTERNATIONAL DE RECHERCHES DERMATOLOGIQUES C.I.R.D.) 27 June 1990 see page 12; example 10 ---	1,3
P,X	CANCER RESEARCH, vol.54, 15 April 1994, BALTIMORE, MD(US) pages 2207 - 2212 K.A. WALTER ET AL. 'interstitial taxol delivered from a biodegradable polymer implant against experimental malignant glioma' Y see page 2210, column 2, line 14 - line 21 ---	1,5, 27-30, 38,39
X	JOURNAL OF MICROENCAPSULATION, vol.7, no.2, May 1990, LONDON (GB) pages 191 - 197 M.-H. BARTOLI 'in vitro and in vivo antitumoral activity of free and encapsulated taxol' A see the whole document ---	31-36
A	WO,A,92 12717 (BREM ET AL.) 6 August 1992 see page 1, line 1 - line 2 see page 9, line 15 - line 30 ---	5
P,X	EP,A,0 567 816 (BEHRINGWERKE AKTIENGESELLSCHAFT) 3 November 1993 see claims 1,3,4,18,19 ---	25,26
Y	WO,A,92 15286 (NOVA PHARMACEUTICAL CORPORATION) 17 September 1992 see the whole document -----	1-14
		1,4
		17-23, 31-36

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA94/00373

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 15,16,18-38
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15,16,18-38 are directed to a method of
treatment of the human/animal body, the search has been carried
out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 94/00373

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0470569	12-02-92	CA-A- 2048544 JP-A- 5000969 US-A- 5202352	09-02-92 08-01-93 13-04-93
WO-A-9111193	08-08-91	AU-B- 652744 AU-A- 7335191 EP-A- 0512071 JP-T- 6509791	08-09-94 21-08-91 11-11-92 02-11-94
WO-A-9110424	25-07-91	US-A- 5192744 EP-A- 0463152 JP-T- 4504429	09-03-93 02-01-92 06-08-92
EP-A-0375520	27-06-90	LU-A- 87410 AU-B- 626619 AU-A- 4709989 CA-A- 2006028 DE-T- 68905914 ES-T- 2054069 JP-A- 3135913 US-A- 5292512	10-07-90 06-08-92 28-06-90 20-06-90 14-10-93 01-08-94 10-06-91 08-03-94
WO-A-9212717	06-08-92	AU-A- 1411992	27-08-92
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(54) Title: CROSSLINKABLE POLYPEPTIDE COMPOSITIONS		
(57) Abstract <p>In accordance with the present invention, there are provided rapidly crosslinkable polypeptides which are obtained upon introduction of unsaturated group(s) into the polypeptide via linkage to amino acid residues on the polypeptide directly through one of three types of linkages, namely, an amide linkage, an ester linkage, or a thioester linkage. Each of these linkages are obtainable in a single step by use of a single derivatizing agent, acrylic anhydride. Also provided are methods for preparing such modified polypeptides and various uses therefor. It has unexpectedly been found that proteins with the above-described chemical modifications have the ability to rapidly crosslink to themselves under suitable conditions. This cross-linking occurs in the absence of any external crosslinking agents (indeed, in the absence of any extraneous agents), resulting in the formation of a solid gel material. Solid crosslinked gels are formed in seconds, starting from a freely flowing solution of polypeptide. Applications of such materials are broad ranging, including the encapsulation of living cells, the encapsulation of biologically active materials, the <i>in situ</i> formation of degradable gels, the formation of wound dressings, the prevention of post-surgical adhesions, gene delivery, drug targeting, as a microcarrier for culture of living cells, and the like.</p>		

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CROSSLINKABLE POLYPEPTIDE COMPOSITIONSFIELD OF THE INVENTION

The present invention relates to methods for the modification of polypeptides. In a particular aspect, the present invention relates to modified polypeptides which
5 can readily be crosslinked to produce a gel under extremely mild conditions. Such materials can be used, for example, for encapsulation of biologically active materials, including living cells.

BACKGROUND OF THE INVENTION

10 The crosslinking of proteins by various means has generated much interest in the fields of drug delivery, protein immobilization, enzyme and antibody immobilization, peptide-protein conjugation, vaccines, medical imaging, etc. The applications of such crosslinked protein systems
15 are as diverse as the methods employed to achieve crosslinking.

The use of crosslinked proteins as scaffolds for drug delivery has been pursued by several investigators due to the intrinsic biodegradable nature of proteins *in vivo*.
20 By far the most common method for protein crosslinking is the addition of external crosslinking agents.

Crosslinked protein compositions may take several forms. Microspheres comprising crosslinked proteins are typical in applications that involve drug delivery.
25 Microspheres of proteins are typically prepared by emulsification of an aqueous protein solution with an organic phase and crosslinking by addition of multifunctional crosslinking agents such as glutaraldehyde (Langhein et al., 1987, J. Appl. Bacteriology 63: 443-448;
30 Yan et al., 1988, Biotechnology and Applied Biochemistry 10: 13-20), or by heat denaturation (Law et al., 1991,

Biomat. Art. Cells & Immob. Biotech. 19:613-629; Welz and Ofner, 1992, J. Pharmaceutical Sciences 81:85-90).

Immobilization of proteins on surfaces for enzymatic and chromatographic applications has also been reported in the literature. Proteins and peptides may be immobilized at surfaces by use of crosslinking agents such as glutaraldehyde and carbodiimides (Benslimane et al., 1986, Biomaterials 7:268-72). Preparation of protein-protein or protein-peptide conjugates is commonly performed by use of glutaraldehyde as well as by use of heterobifunctional crosslinking agents such as N-succinimidyl bromoacetate (Bernatowitz and Matsueda, 1986, Anal. Biochem. 155: 95-102). Proteins have also been modified to introduce functional groups that may be polymerized upon exposure to free radicals resulting in the formation of crosslinked hydrogels (Park, 1988, Biomaterials 9:435-441).

Although most of the methods referred to above result in the formation of crosslinked proteins, the use of external agents (and the reaction conditions required for crosslinking) are too toxic for such processes to be carried out in the presence of living cells and tissues. Indeed none of the references noted above teach their respective art in the presence of living systems.

It is well known that agents of crosslinking such as those described above are in fact used as fixatives for cells and tissues. In a slightly different approach from the addition of external crosslinking agents, Park (1988), supra, describes the free radical polymerization of monomers such as acrylic acid and acrylamide along with derivatized proteins as multifunctional crosslinkers for the formation of polyacrylic acid and polyacrylamide gels. In this case the protein, derivatized with unsaturated groups capable of undergoing free radical polymerization,

serves merely as the crosslinker, while the bulk of the resultant hydrogel is either polyacrylic acid or polyacrylamide. The formation of crosslinked hydrogels also necessitates the use of toxic free radical initiators, such as ammonium persulfate, and polymerization conditions that involve temperatures of 60°C as well as polymerization times of an hour or more. No known living cells, except thermophilic organisms, are likely to survive such crosslinking conditions. Thus, in general, the encapsulation of living cells in a crosslinked protein gel has not been described in the art.

In general the encapsulation of cells requires conditions that are particularly fastidious with respect to mild temperatures, absence of toxic chemicals, rigid maintainence of physiological conditions of pH and osmolarity, and processes that in general are fairly rapid so as to minimize the exposure of the living cells to adverse conditions. A good example of a nontoxic encapsulation process is the one using sodium alginate (a polysaccharide) that can be formulated in physiological saline (see, for example, Soon-Shiong et al., 1991, Transplantation Proceedings 23:758). Cells are simply suspended in a solution of polysaccharide, which is added dropwise into a solution of calcium chloride, resulting in the instantaneous formation of capsules of ionically crosslinked alginate containing entrapped cells.

Since proteins, in general, do not spontaneously form gels, external agents must be added to facilitate the formation of crosslinked hydrogels (an exception is gelatin, which can coagulate to form a gel below a certain temperature). The resulting protein hydrogels could potentially be utilized to entrap cells in a crosslinked protein matrix. Thus the solution of a protein may be stirred with an added external crosslinking agent to form a crosslinked protein mass or gel. Alternately the

formation of a protein gel in the form of spheres or microspheres requires emulsification with a nonsolvent phase to form discrete droplets of the protein solution which can subsequently be crosslinked. However, as
5 described above, common processes utilized to crosslink proteins suffer from the limitations of toxicity when contemplated for the encapsulation of living material.

There are several advantages attendant to the use of proteins as encapsulation materials for living cells and
10 tissue. Proteins such as albumin, collagen, gelatin, and the like, being of natural origin, are well tolerated by living cells. For example, the use of albumin in culture media is well known and is in fact essential for the well being of cell cultures. Collagen is secreted by cells and
15 forms the major component of the extracellular matrix. Gelatin is known to support cell adhesive behavior through its binding with fibronectin, another ubiquitous cell adhesion molecule. Thus a matrix of such proteins in the form of a microcapsule is favorable for the growth of the
20 encapsulated cell. In fact commercially available gels such as Matrigel and Atrigel, both of which contain collagen, are known for their ability to support viable cells.

Albumin is considered to be an 'inert' protein
25 since it does not bear epitopes that play a role in cell adhesion under normal physiological conditions. As a result, it does not support cell adhesion and is often utilized as a coating in applications that require a cell-free surface. Thus microcapsules or crosslinked gels of
30 albumin are not expected to show a cell adhesive response when transplanted into a host organism. This effect in general is termed as 'biocompatibility'. Thus in applications such as cell therapy where foreign cells are encapsulated and transplanted to replace lost function in
35 the host, such a 'coating' or encapsulation of the

transplanted cell would prevent an inflammatory and fibrous reaction to the transplanted material. On the other hand, it is often required that transplanted tissue become vascularized or that the material of encapsulation become
5 vascularized so that the encapsulated cells within the matrix of the crosslinked material are in reasonable proximity to a source of nutrients, and, more importantly, to a source of oxygen. In such a case, the use of crosslinked collagen or gelatin would be of great benefit
10 in supporting the growth of vascularized tissue adjacent to the encapsulated cell.

Thus, it is essential to develop protein compositions and processes that can result in the formation of crosslinked protein gels in the presence of living cells
15 in a manner that is innocuous to the well being of the cellular material. The essential requirements of such compositions and processes would be as follows:

-the ability to crosslink in the presence of a suitable initiating system, where the
20 initiating system itself is nontoxic;

-the protein composition should be nontoxic;
-the crosslinking reaction must be
nondetrimental to the cellular material, i.e, it produces little or no heat, it produces no
25 by-products that are harmful to the living material and it does not alter, by chemical reaction, the chemical nature of the encapsulated material; and

-the process must be extremely rapid (it
30 should be complete in a timescale measured in seconds) to avoid prolonged exposure of the encapsulated material to the crosslinking conditions.

The present invention discloses compositions and processes
35 that satisfy each of the above stringent requirements.

In general, the production of crosslinked hydrogels requires the use of water-soluble monomers or macromonomers (in the case where the starting soluble material is a polymer that is subsequently crosslinked).

5 These monomers or macromonomers are dissolved in aqueous medium and suitable agents are added to initiate crosslinking. Crosslinking of proteins is conventionally carried out with addition of crosslinking agents such as those mentioned above. Alternately, the monomers and

10 macromonomers may possess functional groups that are themselves capable of undergoing a crosslinking reaction, without the addition of external crosslinking agents, when subjected to the appropriate conditions. A typical example is the formation of a polyacrylamide gel. The monomer,

15 acrylamide, along with a small amount of bis-acrylamide, is dissolved in an aqueous phase. In the presence of a free radical initiating system, this mixture yields a polymerized crosslinked gel. Another example is the use of a macromonomer, such as polyethylene glycol diacrylate

20 (which is a polyethylene glycol with two introduced acrylate functionalities), which may be dissolved in an aqueous phase. In the presence of free radicals, the acrylate groups polymerize, resulting in a crosslinked hydrogel.

25 The case of proteins, however, presents special problems. The introduction of a free radical polymerizable group (a functional group containing a polymerizable double or triple bond, also known as unsaturation) into the protein molecule is not trivial due to the sensitivity of

30 the protein to its environment. The use of organic solvents and other harsh conditions commonly used to modify synthetic polymers are not possible with proteins due to their denaturation potential. For example, in the synthesis of polyethylene glycol diacrylate (PEG-DA) from

35 PEG, one can use the derivatizing reagent acryloyl chloride. This reagent is extremely reactive and results

in excellent yield of PEG-DA in a dry organic solvent such as dichloromethane or benzene. However, the presence of moisture will rapidly destroy the derivatizing capability of this reagent due to its rapid reaction with water. Such
5 a reagent is clearly unacceptable for modification of protein, given that most proteins will not tolerate organic solvents.

Although it has been reported in the art that a functional group containing unsaturation may be introduced
10 into a protein molecule under relatively mild aqueous conditions, we have found in the course of the present work that this in itself is not a necessary and sufficient condition for the rapid (within seconds) formation of a crosslinked protein gel.

15 BRIEF DESCRIPTION OF THE INVENTION

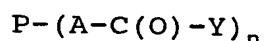
In accordance with the present invention, we have discovered that a rapidly crosslinkable polypeptide may be obtained if, and only if, the unsaturated group introduced into the polypeptide is linked to amino acid residues on
20 the polypeptide directly through one of three types of linkages, namely, an amide linkage, an ester linkage, or a thioester linkage. In addition, each of these linkages are obtainable in a single step by use of a single derivatizing agent, acrylic anhydride.

25 It has unexpectedly been found that proteins with the above-described chemical modifications have the ability to rapidly crosslink to themselves under suitable conditions. This crosslinking occurs in the absence of any external crosslinking agents (indeed, in the absence of any
30 extraneous agents), resulting in the formation of a solid gel material. Solid crosslinked gels are formed in seconds, starting from a freely flowing solution of polypeptide. Applications of such materials are broad

ranging, including the encapsulation of living cells, the encapsulation of biologically active materials, the *in situ* formation of degradable gels, the formation of wound dressings, the prevention of post-surgical adhesions, gene
5 delivery, drug targetting, as a microcarrier for culture of living cells, and the like.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided chemically modified polypeptides having the
10 formula:



wherein:

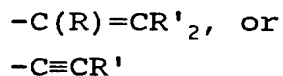
P is any polypeptide,
A is a linking moiety which, in combination with
15 a carbonyl moiety, links Y to P,
Y is an unsaturated group capable of undergoing free radical polymerization, and
n is at least 1.

Virtually any polypeptide can be used in the
20 practice of the present invention, including naturally occurring polypeptides, synthetic polypeptides, short chain peptides having only a few residues, extremely high molecular weight polypeptides, and the like.

The linking moiety "A" employed in the practice
25 of the present invention to link unsaturated group, Y (via a carbonyl) to polypeptide P is typically derived from a reactive residue on the polypeptide backbone. Thus, A is generally selected from -O-, -S-, -NR- or alkylene, or an -O-, -S- or -NR-containing alkylene moiety, wherein R is
30 selected from hydrogen or lower alkyl.

Y of the above formula can be any alkene-containing moiety or alkyne-containing moiety, with

terminal unsaturation preferred because such species are more reactive than internally unsubstituted compounds. Thus, a preferred group of species which are contemplated for use in the practice of the present invention are
5 defined as follows:



wherein:

R is selected from hydrogen, lower alkyl or
10 substituted lower alkyl, and
R' is selected from hydrogen or lower alkyl.

It is preferred that each R' in the above formulae is hydrogen, with Y being $-CH=CH_2$ or $-C\equiv CH$ preferred.

The degree of substitution on polypeptide, P, can
15 vary widely. Typically, n of the above general formula falls in the range of 1 up to about 500, with n in the range of about 2-300 preferred; and n in the range of about 3-100 especially preferred. For many medium sized proteins, n can fall in as narrow a range as 5 up to about
20 60. Of course, those of skill in the art recognize that the desired level of substitution will vary depending on the ultimate use contemplated.

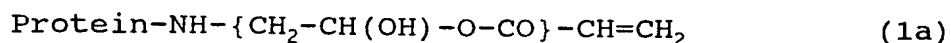
The present invention is based on the observation that polypeptides substituted with unsaturated groups
25 linked to amino acid residues in the polypeptide only through very specific linkages, have the capability of rapidly polymerizing under appropriate free radical initiating conditions to form crosslinked polypeptide gels. More specifically, the invention is based on the ability to
30 very rapidly (in seconds) form crosslinked gels from modified polypeptides starting from a freely flowing solution of polypeptide. Thus, by exposing a polypeptide solution to a suitable wavelength of light (visible or ultraviolet) in the presence of the appropriate

photoinitiators and catalysts, rapid formation of crosslinked gels occurs.

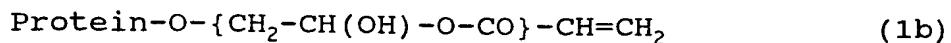
Invention compositions comprise naturally occurring or synthetic polypeptides modified by the substitution of an unsaturated group linked through an amide, ester or thioester linkage to the amines, hydroxyls or sulfhydryl groups, respectively, present on the amino acid residues on the polypeptide. The invention is premised, at least in part, on the unexpected observation that the introduction of a plurality of unsaturated groups into a polypeptide molecule is not the necessary and sufficient condition for rapid formation of a crosslinked gel upon exposure of the polypeptide solution to photoinitiating conditions. In accordance with the present invention, it has been discovered that it is how these unsaturated groups are linked to amino acids (i.e., the particular intervening chemical linkages between the unsaturated groups and the amino acid residues) in the protein that determines the rapidly crosslinkable nature of the resulting substituted protein molecules.

For example, a protein reacted with glycidyl acrylate in aqueous conditions results in vinyl ($-\text{CH}=\text{CH}_2$) substituents, but these vinyls are linked to the protein through intervening groups $\{\text{CH}_2-\text{CH}(\text{OH})-\text{O}-\text{CO}\}$, as indicated below:

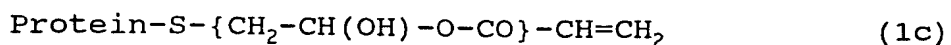
Reaction at lysine residues:



Reaction at serine residues:



Reaction at cysteine residues:

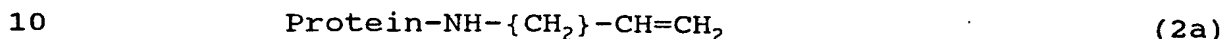


If a solution of the above modified protein with a plurality of such substituents is exposed to conditions of

photoinitiated free radical formation, it will not rapidly crosslink to form a coherent gel.

Another example of the introduction of vinyl substituents into a protein molecule is the reaction of protein with allyl bromide in aqueous media. This results in the following derivative of the protein, in which the intervening group between the vinyl and the amino acid is {CH₂}:

Reaction at lysine residues:



Reaction at serine residues:



Reaction at cysteine residues:



15 If a solution of the above modified protein with a plurality of such substituents is exposed to conditions of photoinitiated free radical formation, it will not rapidly crosslink to form a coherent gel.

However, if a protein or polypeptide is reacted with acrylic anhydride in aqueous media to obtain the following vinyl containing structures, in which the intervening group is {CO}:

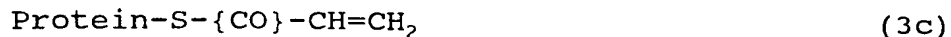
Reaction at lysine residues:



25 Reaction at serine residues:



Reaction at cysteine residues:



it is found that such a protein or polypeptide, having an equivalent degree of substitution of vinyl groups as in the

two examples set forth above will rapidly crosslink (in seconds), resulting in the formation of a coherent gel.

The potential amino acids that are likely to be derivatized by this chemistry are those containing primary or secondary amines ($-\text{NH}_2$ or $-\text{NHR}$, respectively; e.g., lysine, proline, tryptophan, asparagine, glutamine, arginine, histidine), those amino acids containing primary or secondary hydroxyls ($-\text{OH}$; e.g., serine, threonine, tyrosine), and those amino acids containing sulfhydryls ($-\text{SH}$; cysteine). In each of the above cases, due to the reactivity of the reagents with nucleophiles in general, a small fraction of the vinylic substitution occurs on amino acid residues containing hydroxyl groups and sulfhydryl groups. However, due to the general preponderance of lysines (relative to serine and cysteine residues) in proteins, most of the vinylic substitution on the protein is expected to occur at the lysine residues. In addition, glycosylated proteins possessing sugar residues or carbohydrate moieties will also be derivatized due to the reactivity of acrylic anhydride with nucleophiles in general. The latter examples of vinylic substitution (structures 3a, b, c) provide a great unexpected advantage due to the rapid crosslinking capability of the substituents resulting from the reaction of the protein with acrylic anhydride. In other words, a solution of the modified protein of structure 3 when subject to the appropriate photoinitiating conditions, can polymerize in a matter of seconds to a homogeneous crosslinked protein gel.

However, in the cases of modified proteins (or polypeptides) of structures 1 and 2 above, under the same conditions of photoinitiation, protein concentration etc., the reaction does not progress rapidly enough to form a crosslinked protein gel in similar time frames. External monomers such as acrylic acid or acrylamide may be added in

such cases (i.e., for proteins 1 and 2) to cause such a solution to polymerize and form a gel. In such a case, the high reactivity and rapid polymerizing ability of these monomers overrides the lower reactivity of the unsaturated substituents on the proteins (in case of proteins 1 and 2) and in a sense 'kick starts' the reaction to completion. These monomers, however, have to be present at relatively high concentrations for such a reaction to occur (typically greater than 10% by weight of solution). Moreover the severe toxicity of these monomers and large exotherms produced under rapid photoinitiating conditions proves lethal in the presence of living cells and tissue. A good example is the polymerization of cyanoacrylates on tissue, which is well known to cause tissue necrosis.

Thus the present invention describes protein and polypeptide compositions (structures 3 a, b, c) containing unsaturated groups linked directly to amino acid residues in the protein through a {CO} group. These linkages can occur on any amino acid residues that possess primary or secondary amine (e.g., lysines), primary or secondary hydroxyl (e.g., serines) or sulfhydryl (e.g., cysteines) groups. These modified proteins in solution form have the capability to rapidly (within seconds) form a crosslinked protein gel under conditions of photoinitiated free radical generation with no toxicity to living cells or tissue and without the addition of substantial quantities of non-protein free radical polymerizable monomers.

In accordance with one aspect of the present invention, a technique for modification of polypeptides in an aqueous environment that does not result in any adverse effects on polypeptide structure has been developed. Thus, in accordance with the present invention, there is provided a method for preparing chemically modified polypeptides capable of undergoing free radical polymerization. The invention method comprises:

contacting a polypeptide, P, with a reactant containing the group $-C(O)-Y$,

wherein Y is an unsaturated group capable of undergoing free radical polymerization, and

5 wherein said contacting is carried out under conditions suitable to link the moiety $-C(O)-Y$ to P.

The use of the reagent acrylic anhydride as the functionalizing reactant results in substituted proteins that can readily be polymerized under the appropriate free
10 radical generating conditions. Other agents such as allyl bromide or glycidyl acrylate that can also be utilized to functionalize proteins in an aqueous environment do not result in a protein that can rapidly crosslink to form a gel. While not wishing to be bound by any theory, it is
15 presently believed that this most probably arises from the effect of the chemical group immediately adjacent to the vinyl group, i.e., it is the nature of the intervening linkage between the vinyl group and the nucleophilic group on the amino acid that determines the reactivity of the
20 vinyl group in the presence of free radicals. In accordance with the present invention, it has unexpectedly been found that when this linkage is a carbonyl group, i.e., $-CO-$, the reactivity of unsaturated groups to free radicals is greatly enhanced as compared to the cases where
25 groups such as $-CH_2-$ or $-CH_2-CH(OH)-O-CO-$ serve as the intervening linkages. The reagent acrylic anhydride, when reacted with typical nucleophiles in the protein, provides an intervening group between unsaturated groups and the amino acid that is a $-CO-$ linkage. Other reagents which
30 also provide the desired intervening group between unsaturated groups and the amino acid include alkenoic acids or the corresponding acid halides or acid anhydrides thereof, alkylol (meth)acrylamide derivatives, and the like. Presently preferred reactants are alkenoic acid
35 anhydrides. Exemplary reactants include acryloyl chloride, methacryloyl chloride, acrylic acid, methacrylic acid,

acrylic anhydride, methacrylic anhydride, N-methylol acrylamide, N-methylol methacrylamide, and the like.

Any protein that possesses sufficient quantities of nucleophile-containing amino acids can be reacted with acrylic anhydride to produce a rapidly photocrosslinkable material. Most proteins have several lysine residues in the structures that are accessible to modification by this technique. For example, proteins such as albumin, collagen, gelatin, immunoglobulins, hemoglobin, transferrin, caesin, pepsin, trypsin, chymotrypsin, fibronectin, vitronectin, laminin, lipase, hemoglobin, lysozyme, fibrinogen, transferrin, interleukin-1, interleukin-2, tissue necrosis factor, colony-stimulating factor, epidermal growth factor, transforming growth factors, fibroblast growth factor, insulin-like growth factors, hirudin, tissue plasminogen activator, urokinase, streptokinase, erythropoietin, Factor VIII, Factor IX, insulin, somatostatin, proinsulin, macrophage-inhibiting factor, macrophage-activating factor, muramyl dipeptide, interferons, glucocerebrosidase, calcitonin, oxytocin, growth hormone, α -1 antitrypsin, superoxide dismutase, α -2-macroglobulin, lactalbumin, ovalalbumin, amylase, and the like.

In addition to the above-described proteins, polypeptides as short as a few residues (e.g., RGD, YIGSR, REDV, PDSGR, IKVAV, RDGF, GRGD, RGDY, GRGDY, GYIGSR, GYIGSRY, RGDS, GREDV, GREDVY, GRGDF, GPDSGR, GPDSGRY, GIKVAV, IKVAVY, GIKVAVY, and the like) can also be treated in accordance with the present invention. Such polypeptides can be chemically modified by the methods described herein, as well as entrapped within a crosslinked gel of a modified peptide.

The degree of substitution on the protein or polypeptide treated as described herein can be varied quite

easily by using varying molar ratios of acrylic anhydride to the protein/polypeptide (or to the lysines in the protein/polypeptide). Lower substitution results in softer gel formation following the crosslinking reaction compared to proteins with higher degrees of substitution. The former gels are more diffusible than the latter. Thus, adjustment of the degree of substitution allows for the synthesis of a range of materials having from highly diffusible to poorly diffusible crosslinked matrices. The variation of degree of substitution also dictates the rates of *in vivo* degradation of the crosslinked gels. Thus the degradation rate of the material can be tailored to the requirements of the contemplated applications, i.e., short or long degradation times can readily be achieved. Furthermore, the diffusibility of the crosslinked matrix may be tailored to applications such as drug delivery, cell encapsulation, etc.

The photocrosslinking or photopolymerization of modified proteins and/or polypeptides obtained by the modification reaction described herein is performed in the presence of free radical initiating system (e.g., a photosensitizing agent and optionally, a cocatalyst). Typical free radical producing conditions include exposure of the materials to be crosslinked to visible or ultra-violet light. Appropriate photoinitiators and light sources for use can be readily identified by those of skill in the art. In the case of visible light, photoinitiators (also known as photosensitizer or dye) such as ethyl eosin, eosin, erythrosin, rose bengal, thionine, methylene blue, riboflavin may be used. Most visible light photoinitiators require the presence of a cocatalyst (also known as cosynergist, activator, initiating intermediate, quenching partner) to generate the free radicals necessary for polymerization of unsaturated substituents. Examples of such cocatalysts are triethanol amine, methyl diethanol amine, triethylamine, arginine, and the like. Optionally,

the addition of a small quantity of a comonomer (e.g., 1-vinyl 2-pyrrolidinone, acrylamide, methacrylamide, acrylic acid, methacrylic acid, sodium acrylate, sodium methacrylate, hydroxyethyl acrylate, hydroxyethyl methacrylate (HEMA), ethylene glycol diacrylate, ethylene glycol dimethacrylate, pentaerythritol triacrylate, pentaerythritol trimethacrylate, trimethylol propane triacrylate, trimethylol propane trimethacrylate, tripropylene glycol diacrylate, tripropylene glycol dimethacrylate, glyceryl acrylate, glyceryl methacrylate, and the like) can aid in increasing the overall rate of the polymerization reaction. In the case of UV light, photoinitiators that absorb in the UV range such as 2,2-dimethyl phenoxyacetophenone, other acetophenones, benzophenones and their ionic derivatives (for water solubility), benzils and ionic derivatives, thioxanthenes and ionic derivatives, and the like, may be utilized.

In accordance with a particular aspect of the present invention, there are provided articles comprising crosslinked, chemically modified polypeptides as described herein having biologically active material entrapped therein. A wide range of biologically active materials are contemplated for use herein, including peptides, proteins, enzymes, hormones, cytokines, nucleic acids, drugs, and the like. Not only can the chemically modified polypeptides described herein be employed to entrap biologically active material therein, in addition, the polypeptide employed for encapsulation can itself impart physiological activity to the resulting article.

The application of such rapidly crosslinkable polymers is clearly multifold, ranging from the use in encapsulation of cells, microcarrier cultures, drug delivery from a biodegradable scaffold, targeted delivery of drugs, genes, vaccines, and the like, the prevention of

post surgical adhesions, a scaffold for artificial skin, etc. Some of these applications are outlined below.

The rapidly crosslinkable nature of the modified proteins/polypeptides of the invention allow for the formation of crosslinked gels within seconds of exposure to appropriate photoinitiating conditions. The nontoxic nature of the crosslinking reaction allows for entrapment of living cells in the crosslinked matrix. Such a crosslinked matrix may take several geometrical forms such as spheres, sheets, blocks, cylinders, disks, etc., depending on the end use contemplated. The viability of cells under such crosslinking conditions has clearly been demonstrated in the course of the present work.

The crosslinking of proteins (or polypeptides) in the presence of living cells, without toxicity thereto, has not been previously demonstrated in the art. Typical emulsification processes can be utilized in conjunction with the modification processes described herein to generate microspheres in the submicron size range. Photopolymerization of the resulting submicron particles produces submicron particles of crosslinked proteins and/or polypeptides. Such microparticles have applications in a wide variety of fields, e.g., in drug delivery, gene therapy, diagnostic imaging, and the like. In a specific aspect, microspheres prepared under controlled low shear conditions can be utilized for cell encapsulation. Such microspheres may be a few microns to several hundred microns, depending on the cell types encapsulated.

For example, hepatocytes encapsulated in photocrosslinked albumin microspheres can be used as a detoxification system for patients in liver failure. Thus, plasma from the patient is perfused over a bed of encapsulated hepatocytes in order to detoxify or metabolize the accumulated toxins in the patient's blood. In

addition, in the case of liver failure, activated carbon (or charcoal), which is commonly used as an adsorbent for toxins, may also be entrapped in a matrix of crosslinked peptides, such as albumin, and used in a similar fashion.

5 In addition to entrapment of living cells within a crosslinked protein (or polypeptide) matrix, microspheres of crosslinked protein (or polypeptide) may be utilized as a substrate for cell growth. Thus the culture of living cells on microcarriers comprising a crosslinked protein (or
10 polypeptide) matrix is possible. For example, the use of photocrosslinked gelatin beads in the size range of a few microns to several hundred microns may effectively be utilized as a support for living cells such as hepatocytes. Gelatin is known to support cell adhesion on the basis of
15 its affinity for fibronectin, a cell adhesion molecule.

 The use of crosslinked albumin beads as cell growth substrates also provides some interesting opportunities. In general albumin is considered a relatively non-adhesive protein. In fact, several groups
20 have demonstrated that coating of surfaces with albumin prevents cell adhesion. This effect could be utilized to advantage in the case where ligand specificity to cell adhesion is to be elucidated. In such a case, a cell non-adhesive substrate is required upon which can be
25 introduced a ligand with specific interactions for the cell type. Thus in the case where the cell would normally not adhere to an albumin substrate, the specific ligand introduced promotes cell interaction with the modified surface. Albumin microcapsules may be easily modified with
30 such ligands to test specific cell-surface interactions.

 The immobilization or entrapment of drugs within matrices of crosslinked proteins (or polypeptides) has several applications in the field of drug delivery. In accordance with the present invention, the release profile

of drugs from a crosslinked protein (or polypeptide) matrix may be varied by adjusting a number of parameters. Such parameters include the degree of substitution of the protein with unsaturated groups, the concentration of the protein, the loading of the drug, and the like. In addition, the rate of degradation of the protein matrix would also dictate the profile of the released drug. Thus a number of parameters may be manipulated to achieve a desired release profile of a drug.

Microcapsules of crosslinked protein (or polypeptide) with diameters less than 5 microns are suitable for intravenous injection. Such microcapsules containing an entrapped drug can be utilized for intravascular drug delivery. It is known that particulates injected into the blood stream in the micron and submicron size range are scavenged by the reticulo-endothelial system (RES) of cells in the liver and spleen. Crosslinked protein (or polypeptide) microcapsules according to the present invention, containing entrapped drug, will be taken up in these organs and degraded, allowing release of the entrapped drug. Thus, the degradation of these capsules over time should result in a sustained release profile for the encapsulated drug. Several drugs may be contemplated as being useful for delivery in a matrix of crosslinked protein (or polypeptide). Examples of such drugs include analgesic agents (e.g., acetaminophen, aspirin, ibuprofen, morphine and derivatives thereof, and the like), anti-asthmatic agents (e.g., azelastine, ketotifen, traxanox, and the like), antibiotics (e.g., neomycin, streptomycin, chloramphenicol, cephalosporin, ampicillin, penicillin, tetracycline, and the like), anti-depressant agents (e.g., nefopam, oxypertine, imipramine, trazadone, and the like), anti-diabetic agents (e.g., biguanidines, hormones, sulfonylurea derivatives, and the like), anti-fungal agents (e.g., amphotericin B, nystatin, candicidin, and the like), anti-hypertensive agents (e.g.,

propanolol, propafenone, oxyprenolol, nifedipine, reserpine, and the like), anti-inflammatory agents (e.g., steroidal (e.g., cortisone, hydrocortisone, dexamethasone, prednisolone, prednisone, fluazacort, and the like) and
5 non-steroidal (e.g., indomethacin, ibuprofen, ramifenizone, piroxicam, and the like) agents, anti-neoplastic agents (e.g., adriamycin, cyclophosphamide, actinomycin, bleomycin, duanorubicin, doxorubicin, epirubicin, mitomycin, methotrexate, fluorouracil, carboplatin,
10 carmustine (BCNU), cisplatin, etoposide, interferons, phenesterine, taxol (as used herein, the term "taxol" is intended to include taxol analogs and prodrugs, taxanes, and other taxol-like drugs, e.g., Taxotere, and the like), camptothecin and derivatives thereof (which compounds have
15 great promise for the treatment of colon cancer), vinblastine, vincristine, tamoxifen, and the like, anxiolytic agents (e.g., dantrolene, diazepam, and the like), immunosuppressive agents (e.g., cyclosporine (CsA), azathioprine, mizorobine, FK506, prednisone, and the like),
20 physiologically active gases (e.g., air, oxygen, argon, nitrogen, carbon monoxide, carbon dioxide, helium, xenon, nitrous oxide, nitric oxide, nitrogen dioxide, and the like, as well as combinations of any two or more thereof), as well as other pharmacologically active agents, such as
25 cimetidine, mitotane, visadine, halonitrosoureas, anthracyclines, ellipticine, benzocaine, barbiturates, and the like. In addition, drugs in encapsulated or liposomal form may also be entrapped in a matrix of crosslinked protein.

30 In addition to encapsulation of drugs, peptides, hormones, proteins, nucleic acid constructs (e.g., IGF-1 encoding sequence, Factor VIII encoding sequence, Factor IX encoding sequence, antisense nucleotide sequences, etc.), enzymatically active agents (e.g., DNase, ribozymes, and
35 the like), immunostimulating agents (i.e., vaccines, and the like) may be encapsulated or entrapped into the matrix

of a polymerized protein (or polypeptide) gel (such as an albumin gel) and injected intravenously (if the particle size is suitable) or administered by subcutaneous or intrathecal injection. The degradable gel is eventually
5 absorbed while the entrapped hormone or peptide is released over time. This possibility has been demonstrated for insulin release over time (see Example 15). Clearly a plethora of such active agents may be delivered by this technique. Any of the above-described compositions are
10 useful for the treatment of disorders which relate to hormone-deficient disease states. Examples of other deliverable agents include agents employed for the treatment of carcinoma, wound healing, erythropoiesis stimulation, stimulation of fibrinolysis, treatment of
15 hemophilia, glucose regulation, immunoregulation, treatment of Gaucher's disease, treatment of bone disease, induction of labor, treatment of dwarfism, treatment of AAT deficiency, treatment of respiratory disorders, and the like.

20 Crosslinked gels of degradable, non-immunogenic proteins such as albumin, collagen etc. may be injected subcutaneously for cosmetic applications. Injections of collagen are quite commonplace in the field of cosmetic and plastic procedures. The problem with collagen injections
25 is the rapid absorption of the protein after injection. A polymerized/crosslinked gel of collagen or albumin may be injected, and would be expected to degrade over much longer periods of time than do unmodified proteins. Such longer lasting 'implants' may decrease the need for frequent
30 procedures.

In addition to the non-specific uptake of crosslinked protein microspheres by the RES following intravascular administration of these microspheres, specific receptor-ligand interactions between proteins
35 and/or other moieties on the surface of the microspheres

and cellular receptors may be exploited for the purpose of targeted delivery of the protein microspheres. An example is the possibility of specific uptake of microspheres into hepatocytes. The presence of a receptor for polymerized human serum albumin (PHSA) on the surface of hepatocytes has been demonstrated by several research groups (Trevisan et al., 1982, *Hepatology* 2:832-835; Michalak and Bolger, 1989, *Gastroenterology* 96:153-66). It has also been established that such a receptor exists on the surface of the hepatitis B virus (HBV) associated with the HBV surface antigen (HBsAg) (Hansson and Purcell, 1979, *Infect. Immunol.* 26:125-130; Imai et al., *Gastroenterology* 1979, 76:242-247). Imai proposed that PHSA may act as a bridge between the virus and the target liver cells thus explaining the restricted host and organ tropism of HBV infection. PHSA, a macromolecule of approximately 400,000 daltons, as well as antibodies to PHSA have been detected in human plasma, particularly in patients with chronic liver diseases (Lee et al., 1987, *Hepatology* 7:906-912). In normal individuals, the presence of PHSA in the circulation maybe a result of the normal aging process of albumin in human serum, such as excessive oxidation and crosslinking via cysteine residues, and the PHSA receptor on hepatocytes may function as the clearance terminal for PHSA as part of the system for albumin homeostasis *in vivo*. It has been demonstrated that albumin polymerized with glutaraldehyde can bind to these receptors on the hepatocyte (Michalak and Bolger, 1989), supra.

Without wishing to be bound by any theory, it is proposed, based on the evidence presented herein, that albumin, polymerized and/or crosslinked via unsaturated groups incorporated into the protein molecule, would bind to this receptor on the hepatocyte and serve as a targeting moiety for the delivery of pharmacologically active agents to the hepatocyte when the agents are carried along with the crosslinked albumin. Moreover, exploitation of the

binding of PHSA to HBsAg could be utilized to remove significant titers of circulating HBV from blood by contact with a bed of crosslinked albumin particles. In addition, the delivery of genes to hepatocytes for the treatment of
5 genetically deficient states such as hemophilia may be of great benefit. Encapsulation of the genes for Factor VIII production and subsequent delivery directly into the hepatocyte may result in the integration of this gene into the genome of the hepatocyte, resulting in the production
10 of Factor VIII. Several genetically deficient disease states may avail of this methodology employing microcapsule formulations for the delivery of genes to specific sites.

Another example is the delivery of drugs or genetic material to the lungs in an aerosolized
15 formulation. Crosslinked microspheres of proteins in the 1-5 micron size range would be effective in delivery of entrapped or encapsulated pharmacological agents to the lungs. For example anti-inflammatory agents such as ibuprofen or indomethacin may be inhaled directly into the
20 lungs for the treatment of cystic fibrosis. These drugs have been shown to have significant alleviating effects from the disease (Konstan et al., in New England J. Med., 1995, 848-854).

Another application of rapidly photocrosslinkable
25 proteins (or polypeptides) lies in the prevention of post-operative or post-surgical adhesions. Post-operative adhesions, or filmy connective or scar tissue bridges formed during the normal healing process following surgery, often result in bowel obstructions and infertility arising
30 from kinking of fallopian tubes following abdominal surgery. The isolation of wounded tissue (as a result of surgery) by use of a physical barrier of biocompatible, degradable material between this tissue and the surrounding organs has been shown to alleviate these problems. Viscous
35 solutions of hyaluronic acid (HA, a polysaccharide) have

been used previously for this purpose, albeit in a soluble form. As expected, even these fairly viscous solutions of HA are likely to dissolve away, resulting in the eventual formation of adhesions. The use of *in situ* photocrosslinkable solutions of a protein such as albumin, resulting in the formation of a cohesive gel around the injured tissue, is likely to efficiently isolate the injured tissue from surrounding organs and thus prevent the formation of adhesions. The use of crosslinkable albumin, a protein that does not elicit an adhesive response from cells and is degradable *in vivo* to harmless by-products, is advantageous over the use of synthetic materials *in vivo*. Mixtures of photocrosslinkable albumin with hyaluronic acid or a modified photocrosslinkable hyaluronic acid, when polymerized *in situ*, also serves as a degradable barrier to prevent the formation of adhesions.

The ability to polymerize proteins such as albumin, gelatin, collagen etc. into crosslinked hydrogels extends the possibility of use of these materials into the field of wound dressings and skin substitutes. Crosslinked sheets of these protein hydrogel materials may be used as a substrate for the growth of skin cells such as dermal fibroblasts, keratinocytes, and the like. In general, it is problematic to obtain adhesion of cells to a high water content hydrogel. However, the invention compositions allow for substantial amounts of collagen and/or gelatin to be polymerized and crosslinked directly into the matrix of the crosslinked hydrogel. In addition, unmodified collagen or gelatin may be added to the matrix of the above crosslinked hydrogel to enhance and support cell growth.

Collagen is known to support cell adhesion/anchorage through integrin interactions with cells and gelatin most likely supports cell adhesion through its affinity for fibronectin, a cell adhesion molecule which also interacts with integrin. The required cell types can

thereby be cultured on a crosslinked hydrogel matrix sheet containing crosslinked modified albumin, collagen, gelatin etc. in any combination. This sheet of hydrogel and cultured cells may be placed (cell side down) onto wounds
5 such as third degree burns to aid in regeneration of lost skin. The cells may be taken from the patient for autologous transplantation to avoid the problems of graft rejection. Allograft and xenograft cells may also be used for this purpose. In addition, the crosslinked hydrogel,
10 being an absorptive matrix, can be loaded with media, nutrients and growth factors essential to the survival of the cultured cells and factors that aid in the proliferation of the patient's skin cells.

Such a crosslinked hydrogel matrix may also be
15 used as a support for the growth of autologous keratinocytes for gene delivery to a topical site. Progenitor or immature basal keratinocytes may be transfected with a gene cultured on the crosslinked hydrogel substrate, then grafted onto the skin. This
20 results in grafted cells that produce a desired gene product in the host. If necessary, at a later time, the cells can be removed by a simple dermal abrasion procedure.

It has been demonstrated that a wound heals faster in the presence of adequate oxygenation than in
25 cases where a lower concentration of oxygen is available to the proliferating cells in the wound bed. In order to make a wound dressing from a crosslinkable protein as described above with a high permeability to oxygen, several approaches may be satisfactory. For example, the addition
30 of a fluorocarbon emulsion into the protein solution followed by polymerization into a hydrogel sheet would increase permeability to oxygen. Microcapsules of hemoglobin that reversibly bind oxygen have been developed (see PCT publication no. WO 94/18954) that could be
35 incorporated into the dressing as a transporter of oxygen

to the wound bed. In addition, growth factors, growth stimulants, antibiotic drugs, and the like, can easily be incorporated into the wound dressing during the polymerization phase.

5 The crosslinked degradable gels of the invention may be used as a scaffold for modelling of tissue growth *in vitro* or *in vivo*. For example, a degradable protein gel may be utilized as a scaffold for tissue growth and modelling, e.g., for the formation of leaflets of heart
10 valves. In such case, a suitable cell type is grown on the scaffold to establish a three-dimensional tissue-like morphology while the scaffold degrades over a period of time to generate a tissue structure with the integrity of natural tissue. In addition, these scaffolds may entrap
15 certain factors that promote the growth of particular cell types.

 The modified proteins (and/or polypeptides) of the present invention are not limited to photocrosslinking in a solution containing only the modified protein (and/or
20 polypeptide). In general, the modified protein (and/or polypeptide) may be crosslinked in the presence of other monomers, other crosslinkable proteins, other crosslinkable polypeptides, other unmodified proteins, other unmodified polypeptides, other unmodified polymers, and the like. In
25 the case of crosslinking of modified protein (and/or polypeptides) in the presence of other unmodified polymers, entrapment of these polymers within the matrix of the crosslinked protein (and/or polypeptide) is typically achieved. Depending on the molecular weight of the polymer
30 and the degree of substitution of the modified protein, the polymer may be retained within the crosslinked protein matrix if it is large compared to the 'pores' in the crosslinked protein (or polypeptide) matrix, or it may leave the matrix readily by diffusion if its molecular size

is smaller than the average 'pore' size of the crosslinked material.

In general, the crosslinking of two or more different materials results in an intimate intermingling of polymer chains and the resultant composition or physical state is often known as an interpenetrating polymer network (IPN). The formation of an IPN of a modified protein with the polysaccharide alginate has been investigated as part of the work described herein. Alginates have been utilized in the encapsulation of living cells and tissue due to their inherent ionically crosslinkable nature. This provides for extremely mild and gentle conditions for encapsulation that are particularly favorable for living systems. Alginate gels crosslinked with multivalent cations such as calcium are particularly porous and easily allow diffusion of large macromolecules through the crosslinked alginate matrix. It is beneficial in certain cases to limit this porosity. By addition of suitable quantities of modified albumin according to the invention to a solution of alginate, followed by ionic crosslinking of the alginate and covalent crosslinking of the albumin by free radical photoinitiation, it is possible to obtain a crosslinked matrix that comprises two components; the alginate ionically crosslinked to itself and the albumin covalently crosslinked to itself. The two polymeric components are now intimately intertwined in the crosslinked state without being chemically linked to each other.

It must also be noted that for a particular protein used in the mixture, only a particular range of compositions (i.e., alginate to Protein ratios) are effective for achieving dual ionic and covalent crosslinking properties. This is because at low protein concentrations (relative to alginate) there is not enough protein present to produce enough crosslinks to stabilize

the gel, while at high protein concentrations (relative to alginate), a steric hindrance develops that prevents the alginate from crosslinking (ionically) to itself. Thus an intermediate range or window of concentrations (or ratios) of the two species is required to be determined, for each protein, in order that the resulting mixture will have this dual crosslinking property. Such ratios for alginate and modified albumin have been determined and the resulting solutions utilized for the encapsulation of cells (see Example 20). Any modified protein may be utilized in this method along with any desired polymer, natural or synthetic.

The modified proteins of the invention may also be crosslinked in the presence of other monomers or macromonomers that can undergo free radical polymerization to form crosslinked polymeric materials. For example, modified gelatin may be copolymerized with monomers such as acrylic acid, acrylamide, methacrylamide, methacrylic acid, sodium acrylate, sodium methacrylate, hydroxyethyl acrylate, hydroxyethyl methacrylate, vinyl pyrrolidinone, ethylene glycol diacrylate, ethylene glycol dimethacrylate, pentaerythritol triacrylate, pentaerythritol trimethacrylate, trimethylol propane triacrylate, trimethylol propane trimethacrylate, tripropylene glycol diacrylate, tripropylene glycol dimethacrylate, glyceryl acrylate, glyceryl methacrylate, and the like, to form crosslinked materials. The modified proteins may also be copolymerized with macromonomers such as polyethylene glycol acrylates, polysaccharides substituted with free radical polymerizable groups to generate novel classes of polymeric materials. The advantages of such systems lie in the ability to combine into a single composition, the diverse, unique and advantageous properties of the component materials. An example is the use of alginates in combination with the modified proteins as outlined above.

Another example is the copolymerization of a protein such as modified albumin with polyethylene glycol acrylates to generate microcapsules that are extremely biocompatible, i.e., they resist cellular-cellular
5 adhesion. Polyethylene glycol is well known for its ability to resist protein adsorption and cellular adhesion (Desai and Hubbell, 1992, Biomaterials 13:505). For example, PEG bound to bovine serum albumin has shown reduced immunogenicity and increased circulation times in
10 a rabbit (Abuchowski et al., 1977, J. Biol. Chem. 252:3578). Such microcapsules would show long circulation times *in vivo* when injected intravascularly and resist uptake by the RES. Applications for such systems would include drug delivery, diagnostic imaging, gene therapy,
15 and the like.

In addition, surfaces of photocrosslinked protein (or polypeptide) microspheres may be modified with suitable ligands such as antibodies, carbohydrate moieties, and the like, that would be recognizable through specific
20 interaction at the receptor level. This would allow for targeting of these crosslinked microspheres.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

25 Synthesis of Acrylic Anhydride

Acrylic acid (0.2 mol) was reacted with acetanhydride (0.1 mol) at a temperature of 60-70°C for 2 hours. Finely powdered copper (0.1 g) was added as a polymerization inhibitor. The mixture was then vacuum
30 distilled and three separate fractions collected. The first fraction gave predominantly acetic acid, a reaction product, the second fraction gave a mixture of acetic acid and acrylic acid, and the last fraction with a boiling

point of approximately 65°C at 10 mm Hg was predominantly acrylic anhydride. Purity of the fractions was determined by Fourier Transform Infrared Spectrometry. Yield: 60%.

Example 2

5 Synthesis of a Polymerizable Albumin Derivative

Human Serum Albumin (5 g) was dissolved in 100 ml of water and cooled to 4°C in an ice bath. Acrylic anhydride (4 ml) was added drop by drop with constant stirring to the cold protein solution and the pH maintained at 9.0 by addition of suitable quantity of 50% NaOH. The stirring was continued for 24 hours at a temperature of 4°C. The reaction product was neutralized and dialyzed against deionized water through a dialysis membrane with a molecular weight cutoff of 12,000-14,000 for 24 hours. The dialysed product was freeze dried to obtain the protein derivative. Yield: 3.5g. The substitution of vinyl groups by this method was targeted to predominantly the lysine amines present in the protein molecule.

General reaction scheme:

20 Albumin-NH₂ $\xrightarrow{\text{Acrylic anhydride}}$ Albumin-NH-C(O)-CH=CH₂
 Degrees of substitution may be varied. Small amounts of substitution also occur on amino acids possessing hydroxyl and sulfhydryl groups by the following reactions:

25 Albumin-OH $\xrightarrow{\text{Acrylic anhydride}}$ Albumin-O-C(O)-CH=CH₂
 Albumin-SH $\xrightarrow{\text{Acrylic anhydride}}$ Albumin-S-C(O)-CH=CH₂

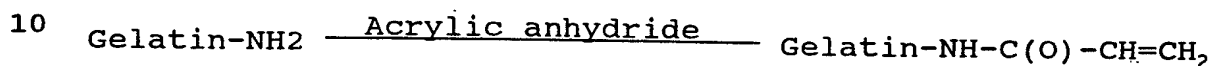
Example 3

30 Synthesis of a Polymerizable Gelatin Derivative

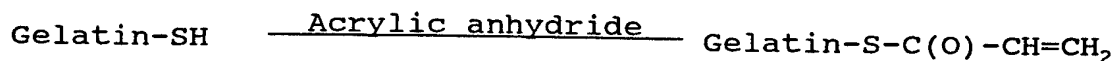
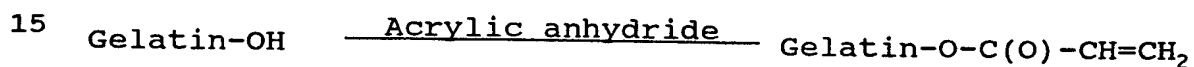
Bovine Gelatin (5 g) was dissolved in 100 ml of water at 40°C. Acrylic anhydride (4 ml) was added drop by drop with constant stirring to the protein solution and the pH maintained at 9.0 by addition of suitable quantity of

50% NaOH. The stirring was continued for 24 hours at a temperature of 40°C. The reaction product was neutralized and dialyzed against deionized water through a dialysis membrane with a molecular weight cutoff of 12000-14000 for 24 hours. The dialysed product was freeze dried to obtain the protein derivative. Yield: 3.5g. The substitution of vinylic groups by this method was targeted to predominantly the lysine amines present in the protein molecule.

General reaction scheme:



Degrees of substitution may be varied. Small amounts of substitution also occur on amino acids possessing hydroxyl and sulfhydryl groups by the following reactions:



Example 4

20 Laser/Visible Light Photopolymerization
to Produce Crosslinked Protein Gels

Substituted proteins prepared by the techniques outlined above were dissolved in aqueous bicarbonate buffered saline (or other buffer) at pH 7.4 at a concentration of 1.0 - 40 % (w/v). A free radical initiating system comprising a dye and a cocatalyst were used to initiate polymerization. The dye, ethyl eosin (0.1mM to 0.1M), a cocatalyst, triethanolamine (0.1mM to 0.1M), and optionally, an accelerator (for increasing the rate of polymerization), vinyl pyrrolidinone (0.001 to 10.0%) were added to the solution which was protected from light until the photopolymerization (alternative choices for the initiator, cocatalyst, and wavelength of laser

radiation are possible). A small volume of solution was placed in a petri dish and exposed to visible radiation from an argon ion laser at a wavelength of 514 nm at powers between 10mW to 1W. An exposure time as low as 100 msec
5 was found to be adequate for polymerization. Photopolymerization was also performed with a mercury arc lamp having a fairly strong emission around 514 nm.

Example 5

UV Photopolymerization to Produce Crosslinked Protein Gels

10 A different initiating system from the one in the example above was used to produce protein gels. A UV photoinitiator, 2,2-dimethoxy-2-phenyl acetophenone dissolved in dimethyl sulfoxide, was added to a solution of substituted protein in aqueous buffer at a concentration of
15 50 - 5000 ppm. This solution was exposed to long wave UV radiation from a 100 watt UV lamp. The time required for gellation was typically less than 30 seconds although this could vary between 1 and 100 seconds depending on the concentrations of initiator and addition of accelerators
20 such as vinyl pyrrolidinone (0.001 to 10.0%). A UV laser may also be used for the photopolymerization.

Example 6

Degree of Modification measured by Amine assay

The percentage of lysine amines modified by
25 reaction of the albumin (could be any protein) with acrylic anhydride could be measured by a simple spectrophotometric assay. The free amines in the protein could be titrated against 2,4,6-trinitrobenzene sulphonic acid (TNBS) which shows an increase in absorption at 420 nm upon reaction
30 with a primary amine. Unmodified albumin was utilized as the control and its absorption after reaction with TNBS was measured at 420 nm. The percent of amine groups on the protein substituted by reaction with varying amounts of

acrylic anhydride was determined by this assay. Substitution from 0.1% to 99.9% of all amines in the protein was possible as measured by this assay. The table below shows the varying percentages of amine substitution for reactions of acrylic anhydride (AA) with albumin lysines for varying molar ratios of AA to lysines present on the protein:

	AA/Lysine molar ratio	% Lysines Substituted
10	0.00	0.0
	0.43	22.2
	3.40	66.1
	8.50	93.2
	12.80	97.6

15

Example 7Comparison of Polymerization Times for other
Vinyl Substituted Proteins

Albumin was reacted with allyl bromide to obtain the structure (2a-c)) shown earlier. Albumin was also reacted with glycidyl acrylate to obtain the structure (1a-c) shown earlier. A solution of 15% (w/v) of these derivatives in water were subject to the polymerization test described in Example 4, in direct comparison with the albumin derivative obtained by reaction with acrylic anhydride. The time required to form a coherent gel in each case was noted. Results are tabulated below:

	Albumin Derivative with:	Polymerization time to coherent gel
30	Glycidyl Acrylate	poor gel > 8 min
	Allyl Bromide	poor gel > 8 min
	Acrylic Anhydride	10 sec

It can be noted upon review of the results tabulated above that a coherent gel was produced only from the protein that had been modified with acrylic anhydride. Other modifications to the protein that result in the
5 introduction of a vinylic group produced gels that were much softer with very poor consistency and at much longer times.

Example 8

Degradation in vivo

10 Crosslinked albumin gels (synthesized from 25% w/v modified albumin solution) were prepared in the form of cylinders of approximately 5 mm diameter. These were equilibrated with Hanks balanced salt solution (HBSS) for two hours prior to implantation in the peritoneal cavity of
15 mice. The disks were surgically implanted into anesthetized mice by simple incision through the peritoneal wall. The disks were weighed prior to implantation. Mice were sacrificed at 1 week, 2 weeks, 4 weeks and 8 weeks, and the crosslinked protein disks were examined for weight
20 loss due to degradation.

No substantial loss in weight was observed when disks were examined at 1 week and 2 weeks. It was estimated that a 10% and 20% loss in weight had occurred at 4 and 8 weeks, respectively, indicating the degradable
25 nature of the crosslinked gel. By manipulating the concentration of the polypeptide employed for crosslinking, and the degree of crosslinking of the gelled material, the rate of degradation of the crosslinked gel can be varied.

Example 9Preparation of Crosslinked Albumin Microspheres
by Emulsification

Human serum albumin modified as above was
5 dissolved in normal saline at a concentration of 10% (w/v).
To the solution were added the photoinitiators and
accelerators as indicated in the above examples. This
solution (1ml) was added to soybean oil (10ml) and stirred
10 rapidly using a magnetic stir bar. After 5 minutes of
stirring when the protein solution was completely
emulsified into the oil, the two phase suspension was
exposed to a 100 watt high pressure mercury lamp for 30
seconds to 5 minutes. The protein solution, now as
discrete droplets in the oil phase was polymerized into
15 discrete crosslinked gelled spheres of diameter typically
less than 100 microns. It was possible to vary this
diameter by controlling the shear during emulsification and
controlling the emulsification time. Normal saline (5ml)
was added to this emulsion and the tube containing this
20 mixture was centrifuged at 3000 xg for 5 minutes. Most of
the microspheres produced separated into the aqueous phase.
The resulting protein microspheres were stored in saline.
Alternate methods of microsphere generation such as
spraying, atomization, sonication, electrostatic droplet
25 generation, coextrusion with air or an oil, etc. followed
by photocrosslinking will result in stable crosslinked
protein spheres.

Example 10Preparation of Crosslinked Gelatin Microspheres
by Emulsification

30

Bovine gelatin, modified as described above (see
Example 3), was dissolved in normal saline at a
concentration of 5% (w/v). To the solution were added the
photoinitiators and accelerators as indicated in the above

examples. This solution (1ml) was added to soybean oil (10ml) and stirred rapidly using a magnetic stir bar. After 5 minutes of stirring when the protein solution was completely emulsified into the oil, the two phase
5 suspension was exposed to a 100 watt high pressure mercury lamp for 30 seconds to 5 minutes. The protein solution, now as discrete droplets in the oil phase was polymerized into discrete crosslinked gelled spheres of diameter typically less than 50 microns. It was possible to vary
10 this diameter by controlling the shear during emulsification and controlling the emulsification time. Normal saline (5ml) was added to this emulsion and the tube containing this mixture was centrifuged at 3000 xg for 5 minutes. Most of the microspheres produced separated into
15 the aqueous phase. The resulting protein microspheres were stored in saline. Alternate methods of microsphere generation such as spraying, atomization, sonication, electrostatic droplet generation, coextrusion with air or an oil, etc. followed by photocrosslinking will result in
20 stable crosslinked protein spheres.

Example 11

Encapsulation of Rat Hepatocytes in Crosslinked Albumin Microspheres

Hepatocytes were isolated from Sprague-Dawley
25 rats by conventional methods of collagenase digestion. The cell pellet (0.2 ml) was resuspended in 1 ml of a solution of 10% modified human serum albumin at physiological pH and osmolarity containing the necessary photoinitiators. This solution was sterile filtered through 0.2 micron filters
30 before use. The cell suspension was added to 10 ml of sterilized soybean oil and gently stirred for 5 minutes before exposure to light as above. Saline was added to the suspension of polymerized albumin droplets containing entrapped hepatocytes and centrifugation at 1000g for 5
35 minutes was performed. The encapsulated cells were

collected from the aqueous phase and cultured. Viability staining with acridine orange-propidium iodide confirmed the viability of these cells at greater than 90%.

Example 12

5 Microcarrier Culture of Rat Hepatocytes on Crosslinked Gelatin Microspheres

Crosslinked gelatin microspheres of diameter 30 - 100 microns were prepared as described above (see Examples 3-5). The spheres were sterilized by ethanol
10 exchange prior to culture use. Hepatocytes were isolated from Sprague-Dawley rats by conventional methods of collagenase digestion. The cell pellet (0.2 ml) was added to a 1 ml pellet of gelatin microspheres and the suspension of cells and microspheres cultured initially in static
15 conditions for 4 hours to allow cell attachment to the microspheres and subsequently in roller bottles. After 24 hours the microspheres were observed under the microscope and substantial adherence of hepatocytes to the gelatin surface was noted. Viability of the cells was greater than
20 90%.

Example 13

Porosity of Crosslinked Protein Gels by Diffusion studies

FITC-Dextran (3 mg/ml) was dissolved in a solution of modified albumin (25% w/v). Solutions (0.5 ml)
25 with added photocatalysts were taken up into a 1 ml syringe. The syringes were exposed to a high pressure Hg lamp to cause rapid polymerization of the modified albumin resulting in entrapment of the dextrans within the resultant gel. This gel could be sheared through a needle
30 attached to the syringe and a known volume of gelled material could be pushed out of the syringe. Such a system is useful for injection of a gel containing entrapped pharmacological agents to be released upon injection.

Two molecular weights of dextran (4400 daltons and 17500 daltons) were chosen as the probes for porosity determination. Cylindrical gels (0.1 ml) were placed in a tube and Hanks buffer (HBSS) was added to the gels. The gels were incubated in HBSS and the supernatant examined periodically in a spectrophotometer for absorbance at 490 nm. From standard curves of absorbance vs. concentration for the FITC labelled dextran, the amount of dextran diffusing out of the microspheres was determined. By utilizing dextrans of different molecular weights (4.4kd, 17.5kd) the relative porosity of the crosslinked hydrogels was estimated. The results are tabulated below:

Time, hours	% Diffusion, 4kd dextran	% Diffusion, 17.5kd dextran
0.016	23.6	6.4
0.25	30.3	11.6
0.5	37.0	14.3
1	45.4	21.2
2	48.7	23.9
3	50.4	29.1
4	55.4	30.0
5	68.8	37.0
6	83.9	47.5

It is seen, upon inspection of the data in the Table, that dextran of molecular weight 4kd diffuses out of the crosslinked gel faster than the dextran of molecular weight 17.5kd, as one might expect. Approximately 85% of the 4kd dextran is released in a 6 hour period in a relatively linear fashion, as compared to ~50% release over the same time period for the 17.5kd dextran. The 4kd dextran approximates the molecular size of insulin, and such a release profile is desired for efficient *in vivo* insulin utilization. See Example 14 for actual experiments with

insulin. By altering the concentration of modified albumin in the starting solution, it is possible to alter the porosity of the crosslinked material, thereby altering the relative rate of release.

5

Example 14In-Vitro Release of Insulin from Crosslinked Albumin Gels

Insulin (Humulin) was dissolved in a solution of modified albumin (25% w/v). Solutions of insulin and modified albumin were made up at concentrations of 5 Units
10 Insulin/ml and 25 Units Insulin/ml each in a 25% albumin solution. The solutions (0.5 ml) with added photocatalysts were taken up into a 1 ml syringe. The syringes were exposed to a high pressure Hg lamp to cause rapid polymerization of the modified albumin resulting in
15 entrapment of the insulin within the resultant gel. This gel could be sheared through a needle attached to the syringe and a known volume of gelled material could be pushed out of the syringe. The gels (0.1 ml) were injected into a tube through a 20G needle and Hanks buffer (HBSS)
20 was added to the gels. The gels were incubated in HBSS and the withdrawn periodically and analyzed for insulin by radioimmunoassay. From standard curves of radioactivity vs. concentration of insulin, the amount of insulin diffusing out of the gels was determined. This information
25 was useful in determining doses for *in vivo* studies. The results are tabulated below. Clearly, by adjusting the porosity of these gels the release of insulin could be controlled so that a longer or shorter acting insulin may be designed.

5	Time (min)	5 Units/ml Gel % Insulin Released	25 Units/ml Gel % Insulin Released
	0	0.0	0.0
	1	5.8	3.7
	5	14.0	37.4
	15	23.2	47.1
	30	30.7	50.4
	60	31.8	51.0
	120	37.5	53.8

10

Example 15In Vivo Release of Insulin from Crosslinked Albumin Gels

Insulin gels prepared as described above (see Example 14), were injected into diabetic rats (made diabetic with streptozotocin) and their blood glucose measured over time and compared to glucose levels in a control diabetic rat receiving conventional soluble form of injectible insulin (Humulin) at a comparable dose. The control rat received 1.5 Units of humulin while the rats that were injected with the gel form of insulin received doses of 1.5 Units and 7.5 Units respectively. The results of blood glucose over time are reported in the Table below.

	RAT#1	RAT#2	RAT#3
	Blood Glucose mg/dl		
	1.5 Units Humulin	1.5 Units GEL	7.5 Units GEL
25	0	496	490
	0.25	462	497
	0.5	405	463
	1	321	417
	2	113	140
	3	159	94
30			48
			43

42

	RAT#1	RAT#2	RAT#3
	Blood Glucose mg/dl		
TIME, Hours	1.5 Units Humulin	1.5 Units GEL	7.5 Units GEL
4	242	74	44
5	428	237	27
6	568	185	127
7	560	219	178
24	440	461	441

It can be seen from inspection of the results tabulated herein that the gel form of insulin was able to maintain lower blood sugar for a longer period in the diabetic rats than the control (commercial injectible insulin). This demonstrated clearly the slow release capability of these gels. In addition, it is known that injection of a high dose of insulin, such as 7.5 Units into a rat is lethal due to hypoglycemic toxicity. The high dose injected in this experiment maintained a low blood sugar without lethality again demonstrating that higher doses may be injected in the gel form as a putative depot form of insulin without the risk of hypoglycemic complications.

Example 16

Coating of Cell Surfaces with Crosslinked Proteins

Due to the rapidly crosslinkable nature of the modified proteins of the invention, it is possible to form thin coatings of crosslinked proteins around the periphery of living cells. Such coatings would be useful in masking the surface antigens of the coated cell thus preventing an immune response if transplanted in a 'non-self' host. The coating would be permeable to relatively small molecules and nutrients while excluding large molecules such as antibodies of the IgG or IgM class that mediate the immune response.

Hepatocytes were exposed to a solution of eosin in saline (0.0005% wt/vol) for five minutes. The cell suspension was centrifuged at 500 g for five minutes and the cell pellet washed twice with saline and centrifuged.

5 The cell pellet was then resuspended in a physiological solution containing 15% (wt/vol) modified albumin and triethanol amine (0.5% v/v). The suspension was exposed to visible light from a high pressure Hg lamp for 2 minutes. As the eosin diffused away from the cells, a thin coat of

10 crosslinked albumin was formed in the region immediately surrounding the cell where all the essential components for the polymerization were present. Excess saline was then added to the suspension whereby the unreacted protein was dissolved and washed away. Following a wash with saline

15 the cells were returned to culture. Coats of crosslinked protein of thickness from a few microns to tens of microns could be obtained by this method. Surface coatings of gelatin or collagen could be prepared by a similar method.

Example 17

20 Microcarrier culture and coating (immunoprotection)

Cells cultured on microcarriers as described above in the case of hepatocytes may be further coated with a layer of crosslinked protein. The method of Example 16 for cell coating can be utilized for coating of

25 microsphere-attached cells. Such a coating would be beneficial not only in protecting the coated cell from an immune response after transplantation but also in extracorporeal devices such as a liver assist device. In such a device, blood from a patient in liver failure is

30 passed through a device where the plasma is separated from the blood cells and passed through a bed of encapsulated or

coated hepatocytes that provide the function of detoxification that is compromised in the patient. The cell coating in this case prevents exposure of circulating antibodies in the patients plasma to the foreign
5 cell-surface antigens that may result in complement activation and subsequent detrimental effects while allowing for exchange and metabolization of circulating toxins.

Example 18

10 Cell coating with gelatin - using charge interactions

Most cell surfaces have a net negative charge due to the presence of glycosylated proteins that typically are present on the exterior of the cell membrane. A positively charged polymer therefore will readily bind to the cell
15 surface through ionic interactions with negatively charged groups. For example a protein such as gelatin type A (net positive charge at neutral pH) will bind to the exterior of the cells. Also synthetic polycations such as polylysine have the same effect. Following the attachment of
20 positively charged polymer or protein at the cell surface (now the cell has a net positive surface charge - this can be determined by electrophoretic mobility or zeta potential measurements) a modified gelatin (with substituted photocrosslinkable groups) with net negative charge can be
25 anchored at this cell surface through charge interactions. The cells are then washed in saline, resuspended in a solution containing the photoinitiators and exposed to visible light when polymerization of the modified gelatin at the exterior of the cell is polymerized to form a thin
30 crosslinked coating. Other modified crosslinkable proteins may be utilized for this method of coating.

Example 19Rapidly Photocrosslinkable Proteins for the Prevention
of Post-Operative Adhesions/Glue

Postoperative adhesions, or filmy connective or
5 scar tissue bridges formed during the normal healing
process following surgery, often result in bowel
obstructions and infertility arising from kinking of
fallopian tubes following abdominal surgery. The isolation
of wounded tissue (as a result of surgery) by use of a
10 physical barrier between this tissue and the surrounding
organs has been shown to alleviate these problems. Viscous
solutions of hyaluronic acid (HA, a polysaccharide) have
been used previously for this purpose, albeit in a soluble
form. As expected, even these fairly viscous solutions of
15 HA are likely to dissolve away, resulting in the eventual
formation of adhesions. The use of *in situ*
photocrosslinkable solutions of a protein such as albumin
resulting, in the formation of a cohesive gel around the
injured tissue, is likely to efficiently isolate the
20 injured tissue from surrounding organs and thus prevent the
formation of adhesions. The use of crosslinkable albumin,
a protein that does not elicit an adhesive response from
cells and is degradable *in vivo* to harmless by-products, is
advantageous over the use of synthetic materials *in vivo*.
25 In addition, combinations of this crosslinkable albumin with
hyaluronic acid and/or crosslinkable hyaluronic acid are
also likely to prevent the formation of adhesion.

Example 20Interpenetrating Polymer Networks of Modified Albumin
and Alginates

Alginates have been utilized in the encapsulation
5 of living cells and tissue due to their inherent ionically
crosslinkable nature. This provides extremely mild and
gentle conditions for encapsulation. Such conditions are
particularly favorable for living systems. Alginate gels
crosslinked with multivalent cations, such as calcium, are
10 particularly porous and easily allow diffusion of large
macromolecules through the crosslinked alginate matrix. It
is beneficial in certain cases to limit this porosity. By
addition of suitable quantities of modified albumin (as
described above) to a solution of alginate, followed by
15 ionic crosslinking of the alginate and covalent
crosslinking of the albumin by free radical
photoinitiation, it is possible to obtain a crosslinked
matrix that comprises two components; the alginate
ionically crosslinked to itself and the albumin covalently
20 crosslinked to itself. The two polymeric components
however are intimately intertwined in the crosslinked state
without being chemically linked to each other. Such a
physical state is called an Interpenetrating Polymer
Network (IPN).

25 It must also be noted that for a particular
protein used in the mixture, only a particular narrow range
of compositions (i.e., alginate to Protein ratios) are
effective to achieve the desired dual ionic and covalent
crosslinking properties. This is because at low protein
30 concentrations (relative to alginate) there is not enough
protein present to produce enough crosslinks to stabilize

the gel, while at high protein concentrations (relative to alginate), a steric hindrance develops that prevents the alginate from (ionically) crosslinking to itself. Thus an intermediate range or window of concentrations (or ratios) of the two species should be determined, for each protein, so that the resulting mixture will have this dual crosslinking property. The following table shows this data for an alginate/albumin IPN:

Alginate/Modified Albumin Ratio*	Physical Property of mixture in 0.4% CaCl_2 **	Physical Property of mixture upon exposure to light 30 seconds***
1 : 1	Coherent gel	No gel
1 : 2	Coherent gel	soft gel
1 : 3	Coherent gel	coherent gel
1 : 4	Soft gel	coherent gel
1 : 5	No gel	coherent gel

* The final concentration of alginate in the mixture (solution containing alginate and modified albumin {80% of lysines modified}) was 1.5%.

** The mixture was dropped into a bath of CaCl_2 through a syringe and observed for spontaneous formation of discrete ionically crosslinked droplets or gelled spheres.

*** The appropriate photoinitiators were added to the mixture and it was exposed to a 100 watt Hg lamp. The mixture was examined for the formation of a crosslinked gel mass.

Thus, mixtures containing alginate and modified albumin that showed gelling under both conditions, i.e., exposure to calcium as well as exposure to light, were considered to be of utility for encapsulation of living material. Thus

ratios in the range of 1:2 to 1:4 were found to be useful for the modified albumin used in this assay.

A mixture of alginate and modified albumin in the useful range, containing the appropriate photoinitiators, was then injected into a solution of calcium chloride (CaCl_2), where discrete droplets (ionically crosslinked) were formed. This suspension of droplets was exposed to visible light from a Hg lamp which caused the ionically crosslinked gel to further be stabilized by covalent crosslinking of the albumin component. This was verified by exposing the dually crosslinked droplets to a solution of sodium citrate (1.0 M, a calcium chelator) following ionic and photocrosslinking. Dissolution or fragmentation of the dually crosslinked gels under these rigorous conditions was considered to reflect a failure of adequate photochemical crosslinking, which would have to be sufficient to stabilize the gel in spite of degelling of alginate caused by sodium citrate. In each case the droplets remained stable in citrate, indicating the presence of a crosslinked network other than that provided by ionically crosslinked alginate. Useful ratios of these mixtures clearly would vary and be dependent on the degree of modification of the albumin as well as the protein utilized in generating the IPNs.

25

Example 21

Encapsulation of Islets of Langerhans in IPN Capsules

Islets of langerhans isolated from dogs, rats, pigs, or humans were obtained by techniques described in the art and maintained in culture. A solution of alginate and modified albumin containing the appropriate photoinitiators was prepared with pH 7.4 and osmolarity 300 mOsm/kg. Prior to encapsulation, the islets were washed in saline and precipitated as a pellet by centrifugation. This pellet was resuspended in the alginate-modified

albumin mixture at a concentration of approximately 5000 islets/ml of solution. This suspension was pumped through a coaxial flow jethead with concentric air flow to produce droplets of a desired size. Droplets produced by this technique were typically 200-700 microns in diameter. The droplets were collected in a beaker containing calcium chloride solution where they instantly gelled by ionic crosslinking on contact with the solution. The transparent glass collection vessel was exposed to a light source (Hg lamp, 100 watt).

The ionically crosslinked capsules were simultaneously polymerized or photocrosslinked upon exposure to light. This resulted in dually crosslinked droplets or capsules containing islets. Exposure of these cells to light was limited to about five minutes, although no evidence of deterioration or damage to islet function was observed at longer times. The capsules were thoroughly rinsed in saline and culture media and then put into culture.

Alternately large capsules (order of mm) could be prepared by injecting the solution through a syringe. Also, microcapsules prepared by conventional techniques could be further encapsulated in a 'macrocapsule' by this technique.

25

Example 22

Encapsulation of Hepatocytes in IPN Capsules

Hepatocytes were isolated from Sprague-Dawley rats by conventional methods of collagenase. A solution of alginate and modified albumin containing the appropriate photoinitiators was prepared with pH 7.4 and osmolarity 300 mOsm/kg. Prior to encapsulation, the hepatocytes were washed in saline and precipitated as a pellet by centrifugation. The cell pellet (0.2 ml) was resuspended

in 1 ml of the encapsulation solution. This suspension was pumped through a coaxial flow jethead with concentric air flow to produce droplets of a desired size. Droplets produced by this technique were typically 200-700 microns
5 in diameter. The droplets were collected in a beaker containing calcium chloride solution where they instantly gelled by ionic crosslinking on contact with the solution. The transparent glass collection vessel was exposed to a light source (Hg lamp, 100 watt).

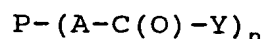
10 The ionically crosslinked capsules were simultaneously polymerized or photocrosslinked upon exposure to light. This resulted in dually crosslinked droplets or capsules containing islets. Exposure of these cells to light was limited to about five minutes, although
15 no evidence of deterioration or damage to islet function was observed at longer times. The capsules were thoroughly rinsed in saline and culture media and then put into culture.

Alternately large capsules (order of mm) could be
20 prepared by injecting the solution through a syringe. Also, microcapsules prepared by conventional techniques could be further encapsulated in a 'macrocapsule' by this technique.

While the invention has been described in detail
25 with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A chemically modified polypeptide having the formula:



wherein:

- 5 P is any polypeptide,
 A is a linking moiety which, in combination with
 a carbonyl moiety, links Y to P,
 Y is an unsaturated group capable of undergoing
 free radical polymerization, and
10 n is at least 1.

2. A polypeptide according to claim 1 wherein P is a naturally occurring or a synthetic polypeptide.

3. A polypeptide according to claim 1 wherein P is selected from albumin, collagen, gelatin, casein, pepsin, trypsin, chymotrypsin, fibronectin, vitronectin, laminin, lipase, hemoglobin, lysozyme, immunoglobulins,
5 fibrinogen, transferrin, interleukin-1, interleukin-2, tissue necrosis factor, colony-stimulating factor, epidermal growth factor, transforming growth factors, fibroblast growth factor, insulin-like growth factors, hirudin, tissue plasminogen activator, urokinase,
10 streptokinase, erythropoietin, Factor VIII, Factor IX, insulin, somatostatin, proinsulin, macrophage-inhibiting factor, macrophage-activating factor, muramyl dipeptide, interferons, glucocerebrosidase, calcitonin, oxytocin, growth hormone, α -1 antitrypsin, superoxide dismutase, α -2-
15 macroglobulin, lactalbumin, ovalalbumin or amylase.

4. A polypeptide according to claim 1 wherein P is selected from albumin, gelatin, collagen or casein.

5. A polypeptide according to claim 1 wherein P is biologically active.

6. A polypeptide according to claim 5 wherein said polypeptide is selected from agents employed for the treatment of carcinoma, wound healing, erythropoiesis stimulation, stimulation of fibrinolysis, treatment of hemophilia, glucose regulation, immunoregulation, treatment of Gaucher's disease, treatment of bone disease, induction of labor, treatment of dwarfism, treatment of AAT deficiency, treatment of respiratory disorders or cosmetic applications.

7. A polypeptide according to claim 1 wherein A is selected from -O-, -S-, -NR- or alkylene, or an -O-, -S- or -NR-containing alkylene moiety, wherein R is selected from hydrogen or lower alkyl.

8. A polypeptide according to claim 1 wherein A is -NR-.

9. A polypeptide according to claim 8 wherein R is hydrogen.

10. A polypeptide according to claim 1 wherein Y is selected from:

-C(R)=CR'₂, or

-C≡CR'

5 wherein:

R is selected from hydrogen, lower alkyl or substituted lower alkyl, and

R' is selected from hydrogen or lower alkyl.

11. A polypeptide according to claim 10 wherein each R' is hydrogen.

12. A polypeptide according to claim 11 wherein Y is -CH=CH₂.

13. A polypeptide according to claim 11 wherein Y is $-C\equiv CH$.

14. A polypeptide according to claim 1 wherein n falls in the range of 1 up to about 500.

15. An article comprising a crosslinked, chemically modified polypeptide according to claim 1 having biologically active material entrapped therein.

16. An article according to claim 15 wherein said biologically active material is selected from peptides, proteins, enzymes, hormones, cytokines, nucleic acids or drugs.

17. An article comprising a crosslinked, chemically modified polypeptide according to claim 1 wherein said polypeptide has physiological activity.

18. An article comprising a crosslinked, chemically modified polypeptide according to claim 5, optionally having biologically active material entrapped therein.

19. A method for preparing chemically modified polypeptides capable of undergoing free radical polymerization, said method comprising

contacting a polypeptide, P, with a reactant
5 containing the group $-C(O)-Y$,

wherein Y is an unsaturated group capable of undergoing free radical polymerization, and

wherein said contacting is carried out under conditions suitable to link the moiety $-C(O)-Y$ to P.

20. A method according to claim 19 wherein said reactant is selected from alkenoic acids or the

corresponding acid halides or acid anhydrides thereof, or alkylol (meth)acrylamide derivatives.

21. A method according to claim 20 wherein said reactant is an alkenoic acid anhydride.

22. A method according to claim 20 wherein said reactant is selected from acryloyl chloride, methacryloyl chloride, acrylic acid, methacrylic acid, acrylic anhydride, methacrylic anhydride, N-methylol acrylamide or
5 N-methylol methacrylamide.

23. A method for covalently crosslinking polypeptides, said method comprising:

contacting a polypeptide, P, with a reactant containing the group $-C(O)-Y$, wherein Y is an unsaturated
5 group capable of undergoing free radical polymerization, and wherein said contacting is carried out under conditions suitable to link the moiety $-C(O)-Y$ to P, and thereafter
contacting the resulting modified polypeptide
with a free radical initiating system under free radical
10 producing conditions.

24. A method according to claim 23 wherein said free radical initiating system comprises a photoinitiating system.

25. A method according to claim 24 wherein said photoinitiating system comprises a photosensitizing agent and optionally, a cocatalyst.

26. A method according to claim 25 wherein said photosensitizing agent is selected from ethyl eosin, eosin, erythrosin, riboflavin, fluorescein, rose bengal, methylene blue, thionine, 2,2-dimethyl phenoxyacetophenone, other acetophenones, benzophenones and their ionic derivatives,

benzils and ionic derivatives, or thioxanthenes and ionic derivatives; and

said cocatalyst is selected from triethanolamine, arginine, methyldiethanol amine, or triethylamine.

27. A method according to claim 23 wherein said free radical initiating system further comprises a comonomer.

28. A method for the delivery of a biologically active agent to a subject, said method comprising administering an article according to claim 15 to said subject.

29. A method according to claim 28 wherein said biologically active agent is selected from peptides, proteins, enzymes, hormones, cytokines, nucleic acids or drugs.

30. A method for the delivery of a biologically active agent to a subject, said method comprising administering an article according to claim 18 to said subject.

31. A method according to claim 30 wherein said biologically active material is selected from peptides, proteins, enzymes, hormones, cytokines, drugs or nucleic acids.

INTERNATIONAL SEARCH REPORT

 International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

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APS, STN

search terms: crosslink, gel, protein, albumin, acrylic, lysine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 4,511,478 A (NOWINSKI ET AL) 16 April 1985 (16.04.85), whole document, especially column 9, lines 20-30.	1-27 ----- 28-31
Y	US 5,204,108 A (ILLUM) 20 April 1993 (20.04.93), whole document, especially column 3, lines 30-45.	1-31

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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P document published prior to the international filing date but later than the priority date claimed	

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(21) International Application Number: PCT/HU98/00086 (22) International Filing Date: 17 September 1998 (17.09.98) (30) Priority Data: P 97 01554 18 September 1997 (18.09.97) HU (71) Applicant (for all designated States except US): HUMAN RT. [HU/HU]; Táncsics út 82, H-2100 Gödöllő (HU). (72) Inventors; and (75) Inventors/Applicants (for US only): HEGEDÜS, Lajos [HU/HU]; Eötvös út 47, H-1121 Budapest (HU). KREM- PELS, Krisztina [HU/HU]; Ady E. út 204, H-1188 Budapest (HU). PAÁL, Krisztina [HU/HU]; Felsőzöldmáli út 80, H-1025 Budapest (HU). PETHŐ, Gábor [HU/HU]; Kassai út 10, H-1225 Budapest (HU). (74) Agent: SOMFAI & PARTNERS; Industrial Rights Co. Ltd., Pozsonyi út 38. II. 5., H-1137 Budapest (HU).		(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HR, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims and statement.</i>
(54) Title: PHARMACEUTICAL COMPOSITIONS CONTAINING PLASMA PROTEIN (57) Abstract The invention is related to water-soluble products and pharmaceutical formulations in solid or liquid form mainly for parenteral use. They consist of or comprise a therapeutically active substance (having low aqueous solubility and a substantial binding affinity to plasma proteins) and a plasma protein fraction in controlled aggregation state, whereby the said active substance and the said protein fraction are bound to each other by way of noncovalent bonds. It also covers processes for the preparation of the product and pharmaceutical formulation.		

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PHARMACEUTICAL COMPOSITIONS CONTAINING PLASMA PROTEIN

The present invention is related to a new method, products and formulations for delivery in therapeutic use of therapeutically active compounds having poor water solubility and substantial binding affinity to plasma proteins and processes for the preparations of such products and formulations.

More particularly first objects of the invention are products and pharmaceutical formulations in solid or liquid form mainly for parenteral use consisting of or comprising

a) a therapeutically active substance having low aqueous solubility and a substantial binding affinity to plasma proteins (in the following "active substance") and

b) a plasma protein fraction in controlled aggregation state

whereby the said active substance and the said protein fraction are bound to each other by way of non-covalent bonds and

c) optionally further pharmaceutically acceptable and mainly parenterally acceptable formulation additive(s) - such as water, stabilizer(s), protein aggregation controller(s).

The homogeneous solid state products of the invention consisting of the said protein and the said substance are water-soluble and their aqueous solutions can be used parenterally or can be used to prepare parenteral pharmaceuticals.

It is well known in the art that some biologically active compounds possess potent therapeutic activity but could never demonstrate their benefit because of their poor solubility in aqueous media. Some of them were never ever formulated while a few did not reach but the stage of the "phase I" clinical development. Some of them appear in "hardly biocompatible" formulations of relatively high toxicity caused by the materials used for formulation. A typical example for this is represented by the groups of taxanes specifically paclitaxel which is a potent cytostatic the application of which however is reduced because of the toxicity of its known formulation in Klucel : tween 80 or Klucel and diluent 12, a 1:1 mixture of Cremaphor EL : ethanol. [Cancer Chemotherapy and Pharmacology (1994) 34:465-471; Journal of the National Cancer Institute (1990) 1247-1259]. Cremaphor EL (polyoxyethylated castor oil) has

inherent toxicity, causing vasodilatation, lethargy, hypotension etc. In order to decrease the toxic side-effect of the solvent and adjuvant, a series of special methods were suggested: application of very small doses over a long period
5 of time, pre-medication before treatment etc. (USP 5665761; USP 5621001; USP 5670537 etc.) A further suggestion consisted in combination of the active substance with a dispersing agent contained within a protein walled shell (USP 5 560 933) which is formed by reacting the protein with oil such as soy
10 bean oil - such formulations being proposed for paclitaxel and amphotericin. However even the latest literature comprises warnings on the course of application of e.g. paclitaxel (see e.g. "Guidance for Industry issued by the U. S. Department of Health and Human Service CDER September
15 1997, OGD-L-8) where - because of hypersensitivity reactions - all patients treated with paclitaxel should be premedicated with corticosteroids, diphenhydramine and H₂ antagonists.

It was further proposed to prepare parenteral
20 formulations of certain water-insoluble dihydropyridins, by dissolving them in an organic solvent or in a mixture of an organic solvent with water and adding an aqueous HSP solution to said solution in order to minimise crystallisation of the insoluble active substance (Hungarian Patent N° 198381; DE
25 Appl. 37 02105). The resulting liquid however was still not a clear solution.

It is further known that some of the water-insoluble active substances possess a considerable affinity to protein or serum protein. Some literature is mentioned
30 here for paclitaxel [Cancer Chem. and Pharm.(1994)34: 465 - 471]; miconazole, fluconazole, amphotericin B [Infection, 23(5): 292 - 297 (1995) Sept.]; carbamazepine [J. Chromatogr. B Biomed. Appl. 669(2): 281 -288 (1995 July 21]; azathioprine [Ann. N.Y. Acad. Sci, 685 (1993): 175 - 192],
35 propofol [J. Chromatogr. Sci (1992): 164 - 166]. According to new literature [The Lancet vol. 352 (1998): 540-542] the drug Taxol® caused rouleaux formation of red cells and so did polyoxyethylated castor oil which served as the solvent of said drug. Some water-insoluble drugs were formulated using
40 the toxic Cremaphor (cyclosporin, teniposide, paclitaxel, amphotericin B). To the best of our knowledge a series of highly active but water-insoluble drugs was not available so

far on the market in parenteral, intravenous administration forms at all e. g. ritonavir, carbamazepine, camphotethine, azathioprine, miconazole, fluconazole etc.

Thus there is a need to solve the problem whereby
5 therapeutically valuable water-insoluble substances can be administered in water-soluble form, preferably parenterally to a patient in need to be treated with said active ingredients.

The aim of this invention is to meet this
10 requirement concerning practically water-insoluble active ingredients having a substantial binding affinity to plasma proteins.

The present invention is based on the recognition that binding the active substances to adequate proteins with
15 non-covalent bonds before administration presents a new and highly potential delivery system for the administration of the active ingredients with poor water solubility. According to the invention homogeneous solid products are produced which are then dissolved in water whereby biocompatible,
20 clear, aqueous solutions are obtained which are suitable for parenteral administration. Thus the invention presents a means to administer the desired water-insoluble active ingredients without introducing the toxic elements and in certain cases in a considerable more efficient dose than
25 before.

Definitions used throughout this application which are henceforth not repeated:

R¹ represents tert. butyl-oxy-carboxylic acid amide or benzoyl amide;

30 R² represents hydrogen or any acyl group preferably acetyl;

Low water-solubility means that the solubility in water at room temperature $< 1 \cdot 10^{-4}$ M;

Substantial binding affinity to plasma proteins means that
35 $>90\%$ of the substance is bound to the proteins in aqueous medium in spontaneous equilibrium at room temperature;

HSA human serum albumin,

WFI water for injection.

One object of the invention is a water-soluble human pharmaceutical formulation mainly for parenteral use containing a therapeutically active compound having low aqueous solubility and a substantial binding affinity to plasma proteins or a human plasma protein fraction in controlled aggregation state.

Other objects of the invention are water-soluble veterinary pharmaceutical formulations mainly for parenteral use containing a therapeutically active compound having low aqueous solubility and a substantial binding affinity to animal plasma proteins in controlled aggregation state.

The human or animal plasmae which can be present in the products and pharmaceutical formulations according to the invention and accordingly used in the methods to prepare the products and compositions can be any of the naturally occurring proteins or plasma fractions such as serum albumin, an immunoglobulin, glycoprotein, interferon and/or interleukin as well as the recombinant analogues of the same. Human and animal proteins can be used. In compounds and compositions intended for treatment of humans the natural human serum and the recombinant human serum proteins are preferred.

The practically water-insoluble active ingredients according to the invention comprise a wide range of compounds whereby the only limitation is that they have to show a substantial affinity to the plasma protein which is selected to be used. Examples for such active ingredients include the following groups of therapeutic agents: a cytostatic such as a taxonoid, antibiotic, vitamin, antiinflammatory, analgesic, anticonvulsant, immunosuppressant, antiepileptic, anxiolytic, hypnotic, antifungal agent, anticoagulant, lipid peroxidase inhibitor, coronary vasodilator, antiarrhythmic agent, cardiotonic, uricosuric, antithrombotic, steroid hormone (progestogen, androgen, testosterone) and/or photosensitizer. Several active ingredients can be used at the same time after careful consideration and adaptation of the therapeutic doses and consideration of the binding affinities to the selected proteins which have to be able to meet such changed requirements.

According to an embodiment of the invention there are provided products and pharmaceutical formulations according

to the above containing at least one of the following active substances: amphotericin B, an adriamidine analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyridamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid and/or warfarin.

A preferred embodiment of the invention consists in a product or formulation as described above containing a taxonoid of the general formula I.

Another preferred embodiment according to the invention contains or consists of paclitaxel and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other human plasma protein fraction.

Further specially important representatives of the invention are homogeneous, solid, water-soluble products consisting of at least one active substance of the group amphotericin B, an adriamidine analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyridamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid and/or warfarin and also consisting of at least one protein of the group human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction where the said active substance and the said protein fraction are bound to each other by way of non-covalent bonds and wherein the molar ratio of the said active substance and the said protein fraction is within the range of 1 : 0.05 to 1 : 100, preferably of 1 : 0.1 to 1 : 50.

Preferred representatives of the above are the following homogeneous, solid, water-soluble products consisting of the following pairs of active substances and proteins:

taxonoid of the general formula I - in the formula

R¹ represents tert. butyl-oxy-carboxylic acid amide or

benzoyl amide,
R² represents hydrogen or any acyl group preferably
acetyl -

and a plasma protein fraction;

- 5 paclitaxel and human serum albumin, recombinant human plasma
albumin and/or γ -globulin;
amphotericin B and human serum albumin, recombinant human
plasma albumin and/or γ -globulin;
10 camptothecin and human serum albumin, recombinant human plasma
albumin and/or γ -globulin;
carbamazepin and human serum albumin, recombinant human plasma
albumin and/or γ -globulin,
cyclosporin A and human serum albumin, recombinant human plasma
albumin and/or γ -globulin;
15 propofol and human serum albumin, recombinant human plasma
albumin and/or γ -globulin.

It is clear from the above explanations that the
invention covers the pharmaceutical formulations as above
both in the solid state and also in the form of the aqueous
20 solutions.

As it is related to their natural structure - more
specifically to their chemical composition - the protein
molecules tend to aggregate through their specific binding
sites. The degree of aggregation depends on the parameters
25 (temperature, composition, relative and absolute
concentration of the components, consequently the pH, ion
strength) of the solution where the protein is present.

The plasma proteins used according to the invention are
preferably in a stabilized or controlled aggregation state.
30 The aim is to avoid such aggregation of the proteins which
would inhibit optimal binding of the active ingredient
actually used. The unwanted aggregation of the proteins can
be controlled by the presence of other molecules capable to
occupy some or all of the binding sites on the macromolecules
35 involved in the aggregation so as to avoid multiple protein -
protein association. Some proteins are available on the
market in a controlled aggregation state: containing
stabilisers to avoid aggregation. This state however is not
always the optimal state for entering into binding with the
40 active substance we intend to use according to the invention.

According to the invention the term "controlled aggregation state" represents the best binding state when the protein is capable to bind the active substance exactly in the manner which is desired for the purpose aimed at. It is not necessarily the state when the maximum number of the active substance molecules are bound to the protein - but there are cases when the highest binding proportion is desirable.

That means that in some cases we have to remove other excipients from e.g. a commercially available serum albumin fraction, such as stabilisers, ionic components, etc. This might be the necessary starting step of the process when the method according to the invention is carried out. The required conditions to establish the proper aggregation stage strictly depends on the actual active substance and the relevant protein fraction.

Examples provided below demonstrate (e.g. paclitaxel and cyclosporine A) that they show a higher binding to a plasma protein fraction in the absence of other excipients (such as stabilisers, ionic components, salts etc.). However there are other active substances (e.g. amphotericin B and propofol) which did not show any interference with the binding of e.g. the protein stabilisers.

Thus the proper aggregation state of the protein used has to be established for each and every pair of active substance / protein which is used according to the present invention.

When using the pair paclitaxel and HSA: it is important to eliminate all stabilisers accompanying commercially available HSA: such as N-acetyl-D,L-tryptophane, alkali caprilates which were used to stabilize the protein during pasteurisation at 60 °C. Amphotericin B or propofol can be bound to HSA also in the presence of these stabilisers. In certain instances, when the desired aggregation state could be reached by water, the other components had to be removed, following e.g. the procedure detailed below in one of the Examples.

The following aspects have to be considered for optimum combination of specific substances with specific plasma protein fractions according to the invention:

a) the characteristics of the binding site occupied by the substance on the protein;

b) possible other components present in the solution occupying the same binding site or even competing for it;

c) the physico-chemical conditions for the conformation of the actual binding site and the consequence to the binding;

d) known therapeutic aspects e.g.

i) paclitaxel on HSA having unic transport characteristics;

ii) paclitaxel on interleukines with proven therapeutic activity of the carrier;

iii) cyclosporin A on gamma immunoglobulin with proven therapeutic activity of the carrier;

iv) ritonavir on gamma immunoglobulin with proven therapeutic activity of the carrier;

stability of the formulation.

One of the simplest aggregation controlling agent is water. Using the proper amount of water unwanted aggregation may be inhibited and the protein is ready to be used according to the invention - it is in "controlled aggregation form".

According to an embodiment of the invention the compounds and compositions may contain as additive a protein aggregation controller or stabilizer and/or solution stabilizing auxiliary additive. Examples for such additives are the following: water, sodium chloride, a buffer, a poly-alcohol such as glycerol, a water-soluble sugar derivative preferably mannitol, sorbitol and/or dulcitol and others.

A further object of the present invention includes the process for the preparation of the new products and the pharmaceutical formulations according to the invention. The process comprises the following steps:

a) dissolving the therapeutically active compound having low aqueous solubility and a substantial binding affinity to plasma proteins ("active substance") in a water-miscible, pharmaceutically acceptable organic solvent,

5 b) combining said solution with the aqueous solution of a plasma protein fraction in controlled aggregation state and optionally

c) a further pharmaceutically acceptable auxiliary additive - such as a protein aggregation controller and/or a
10 stabilizer -

whereby a true solution is obtained containing the said active substance and the said protein fraction bound together by way of non-covalent bonds;

d) removing the organic solvent preferably by
15 ultrafiltering, dialysing, diafiltrating and/or lyophilising the solution or its concentrate or by combination of these treatments

whereby a homogeneous, water-soluble liquid or solid product or pharmaceutical formulation is obtained containing
20 the active substance and the plasma protein fraction;

e) optionally dissolving or diluting the solid or liquid with water whereby a clear, liquid composition is obtained which is suitable for therapeutical administration and

25 f) optionally finishing this product into a parenteral formulation (dosage form) for direct use.

When preparing the new homogenous solid products consisting of the active substances and the proteins bound by way of non-covalent bonds according to the invention it is
30 preferable to use the process comprising the following steps according to the invention:

a) dissolving the therapeutically active compound in a water-miscible, pharmaceutically acceptable organic solvent,

b) combining said solution with the aqueous solution of the selected plasma protein fraction in controlled

5 aggregation state

whereby a true solution is obtained containing the said active substance and the said protein fraction bound together by way of non-covalent bonds;

c) removing the organic solvent and lyophilising the
10 solution or its concentrate.

The proper way to best eliminate the organic solvent depends on the active substance and on the protein involved.

It follows from the nature of the active product (the pair including the active substance and the protein) that the

15 methods applied have to ensure mild conditions.

Lyophilisation leads to homogeneous, solid state water-soluble products which on redissolution in water can be administered intraperitoneally. It might be advantageous to combine the above steps e.g. to make the process more

20 economical by first preparing a concentrate of the active substance/protein pair and thereafter subjecting said concentrate to lyophilisation. Some of the active

substance/protein pairs (e.g. the pair amphotericin B/serum albumin) can be successfully concentrated by way of

25 ultrafiltration or dialysis. Some other pairs (e.g. paclitaxel/HSA) are preferably treated by way of lyophilisation. Some pairs should first be ultrafiltered and the concentrate obtained should then be subjected to lyophilisation.

30 It is clear for the expert in the field that on the course of preparation of parenteral pharmaceuticals dilution with water includes dilution with such aqueous solutions which contain further parenterally acceptable additives such as e.g. sodium chloride.

35 The proper solvent to be used according to the invention to dissolve the active ingredient according to step a) above should have the following properties:

- it should be capable to completely dissolve the active ingredient in its mixture with water and

40 • its mixture with >50% of water should not denaturalize the protein employed.

Before starting to carry out the process according to the invention using the active ingredient and the protein selected the adequate solvent has to be determined on the basis of the above. It is suitable to use solvents where
5 mixtures containing >50% of water are still capable to dissolve the active ingredient.

Preferred solvents which can be used for step a) of the above process are for example any of the group consisting of an aliphatic C₍₂₋₄₎ monoalcohol or polyalcohol, 70 - 100%
10 ethanol, dimethyl formamide, methyl formamide.

When preparing the solution containing the protein an aggregation controller and/or solution stabilizer might be present. Such additives include a further or optimal amount of water. They also include agents capable to partially
15 occupy some of the binding sites of the protein to avoid aggregation such as any of the following agents: sodium chloride, a buffer, a poly- alcohol such as glycerol and/or a water-soluble sugar derivative preferably mannitol, sorbitol, dulcitol.

20 When selecting the optimal conditions in the case of any active ingredient the optimal binding affinities and corresponding aggregation properties have to be determined by preliminary measurements. In the examples below we disclose the full method of such determinations.

25 According to a preferred embodiment of the invention the compounds used in step a) are paclitaxel and a component of the natural plasma such as serum albumin, an immunoglobulin, glycoprotein, interferon and/or interleukin or recombinants of the same are used. Further embodiments
30 according to the invention include to use as the active substance a water-insoluble cytostatic such as a taxonoide, antibiotic, vitamin, antiinflammatory, analgesic, anticonvulsant, immunosuppressant, antiepileptic, anxiolytic, hypnotic, antifungal agent, anticoagulant, lipid peroxidase
35 inhibitor, coronary vasodilator, antiarrhythmic agent, cardiotonic, uricosuric, antithrombotic, steroid hormone (progestogen, androgen, testogen) and/or photosensitizer. Preferred active substances that can be used for the process according to the invention include the following:
40 amphotericin B, an adriamidine analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine,

clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyridamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, 5 ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid and/or warfarin.

A preferred embodiment of the invention consists in the preparation of a homogeneous, solid, water-soluble 10 product consisting of paclitaxel and human serum albumin where the active ingredient and the plasma protein fraction can be in a non-covalent binding. A further preferred embodiment of the invention consists in the preparation of a homogeneous, solid, water-soluble product consisting of a 15 taxonoide of the general formula I and a plasma protein fraction where the active ingredient and the plasma protein fraction are in a non-covalent binding.

It is clear from the above explanations that the present invention is not limited to any of the active 20 substances nor to any of the proteins enlisted above.

A further object of the invention comprises the method of use of the products and formulations according to the invention for treatment of human or veterinary patients. The method consists in administering to a patient in need of a 25 treatment with the active ingredient an effective dose of the composition according to or prepared according to the invention. The doses that have to be applied depend on the active ingredient as well as on the protein used. Doses can be administered to ensure at least the same blood levels 30 which are known to be effective when the specific known active substances are used via other administration routes.

There is provided a preferred method of parenteral treatment of human or veterinary patients with a water-insoluble therapeutically active substance having substantial 35 affinity for binding to plasma protein by way of parenterally administering to a patient in need of a treatment with said active substance an effective dose of the following products preferably using the following dose ranges respectively (calculated on the active substance): 40 paclitaxel/albumin 70 - 280 mg/treatment; propofol/albumin 6 - 10 mg/kg/hour; camptothecin/albumin, gemfibrozil/albumin,

cyclosporin A/ albumin 3 - 5 mg/kg/day; amphotericin B/ albumin up to 1.5 mg/kg/day, whereby the same dose ranges are used for compounds containing the recombinant proteins respectively.

5 The compounds, compositions and methods of the invention present advantages including the following:

- it becomes possible to avoid the use of biologically incompatible vehicles, to diminish or totally avoid dose limiting side effects, related to such components like
10 toxic solvents, surface-active agents, emulsifiers and the like
- the use of plasma protein fractions as drug vehicles presents no additional toxic effects - to the contrary they may improve the tolerance of the patients e.g.
15 in the case of chemotherapy
- in desired cases the applied dose can be increased as compared with the drugs now marketed presenting thus a possibility to improve the overall outcome of therapy.

The present invention is illustrated in a more detailed manner in the following examples without the intention of limitation:

EXAMPLES

5

I. PREPARATIVE METHODS, ASSAYS

The following methods were applied to determine the binding of a particular active ingredient (substance) to a protein:

10

a) Ultrafiltration

15

A 1 ml sample of the clear solution formed by admixture of the aqueous solution containing the protein in controlled aggregation state and the solution of the active ingredient in an appropriate solvent is filtered through an ultrafiltration membrane (cut off limit >30000 Da) and the active ingredient is determined in the ultrafiltrate fraction. When measuring the active ingredient concentration in the unfiltered solution the total amount (>90%) is recovered in unchanged form.

20

b) Lyophilisation

25

1 ml of the above solution is lyophilised. After lyophilization the solid residue is dissolved in about 1.00 ml of distilled water, giving a clear solution. Measuring the active ingredient concentration of this solution no active ingredient is found in the water phase but 100% is recoverable from the protein fraction.

c) Analysis of the Active Ingredient

The assays for the determination of the active ingredient are done by HPLC with detection by UV spectroscopy.

30

The HPLC analysis can be carried out e.g. on a Waters Millennium (Waters, MA, USA) HPLC system. Its components are: Waters 616 pump; Waters 600S controller; Waters 717 plus automatic sample injector, with thermostat set to +5°C; Waters 996 diode array UV/VIS detector. The system is driven and the data acquisition done by Waters Millennium v.2.02.0 run on a Digital P486/166 (Digital Equipments, Irvin, UK) personal computer. The conditions have to be optimised individually for each compound, as exemplified below for several products.

35

d) Proof of the Chemical Structure

40

The LC/MS method is used to prove that the chemical structure of the substance recovered from the bound fraction remained unchanged. The LC/MS assays are performed on a Finnigan Navigator (Finnigan, Manchester, UK) single

quadrupole LC/MS mass spectrometer using the ES or APCI + ionisation mode, with a MassLab v.2.0 data acquisition system run on a Digital Venturis FX/166 (Digital Equipments, Irvin, UK) personal computer. The applied conditions have to be optimised individually for each specific substance, based on the references - as exemplified in several of the following examples.

e.) Preparation of Samples

The following is a typical sample preparation method, used to determine the total concentration/amount of a substance from a sample by HPLC and/or LC/MS analysis.

The solid content of lyophilisation vial is reconstituted with water, the solution is mixed with absolute ethanol in a ratio of 1:1 by volume, precipitating the plasma proteins, while the substances dissolved. After a quick centrifugation, the solution is suitable for HPLC or LC/MS analysis. In LC/MS it is analysed by direct sample introduction or through HPLC by way of separating the components from one another. Both methods give valuable information about the chemical structure of the parent compound and/or the possible degradation products, as exemplified in more detailed manner for several products below.

The chromatographic and mass spectroscopic data from the HPLC and LC/MS studies can confirm the chemical equivalence between the known biologically active substance used as the starting material and the compound recovered after having been bound to a protein fraction according to the invention.

f.) Materials Used

All active substances used were of USP XXIII quality.

The following plasma protein fractions were used in the experiments: (* = Ph. Eur. quality)

Human Albumin 20% sol.*	HUMAN Rt., Gödöllő, Hungary
Recombunin TM 25%	DELTA Biot.Ltd, Nottingham, UK
Humanalbumin 20% *	Biotest Ph., Dreieich, Germany
Albumeon USP	Centeon Bio-Services, Little Rock, AR, USA
Human Albumin 20% Behring*	Centeon Ph. GmbH, Wien, Austria
Human Gamma Globulin 16%*	HUMAN Rt. Gödöllő, Hungary

II. PREPARATION and CHEMICAL or PHYSICAL ASSAYS

In the following examples the plasma protein : substrate binding ratios are in the average range falling between 1 : 0.1 - 100. The substance : HSA binding ratios were calculated based on the assumption for HSA mw = 66500, and human gamma globulin mw = 150000 [see 11 Science, VOL. 244. P.1195-1198, 1989; Vox Sang, 70: p.203-209, 1996]

Example II.1

The 20 % (3.08×10^{-3} M) solution of human serum albumin in controlled aggregation state and the 1 mg/ml (1.17×10^{-3} M) solution of paclitaxel in absolute ethanol were admixed in 4:1 ratio and stirred so as to obtain a clear solution.

The solution is lyophilised; the solid residue is redissolved in sufficient water to ensure a clear solution having the concentration of 20% for human serum albumin . The binding is determined from UF filtrate and retentate fractions, showing 99% binding of paclitaxel to human serum albumin . This represents a 1: 0.1 ratio of human serum albumin : paclitaxel.

Example II.2

The 4.44 % (6.67×10^{-4} M) solution of human serum albumin in controlled aggregation state and the 2.0 mg/ml (2.34×10^{-3} M) solution of paclitaxel (mw 853.92) in absolute ethanol are mixed in a 9:1 ratio and stirred until a clear solution is obtained. The solution is further treated as described in Example II.1.

The binding is determined from UF filtrate and retentate fractions, showing 99% binding of paclitaxel to human serum albumin . This represents a 1: 0.39 ratio of human serum albumin : paclitaxel.

Example II.3

The 4.44 % (6.67×10^{-4} M) solution of recombinant human serum albumin in controlled aggregation state and the 2.0 mg/ml (1.40×10^{-3} M) solution of paclitaxel in absolute ethanol are mixed in 9:1 ratio and stirred obtaining a clear solution.

The solution was lyophilised; the solid residue was redissolved in sufficient water to ensure a clear solution having the concentration of 20% for recombinant human serum albumin. The binding is determined from UF filtrate and

retentate fractions, showing 99% binding of paclitaxel to recombinant human serum albumin. This represents a 1 : 0.24 ratio of recombinant human serum albumin : paclitaxel.

5 **Example II.4**

A 2.25 % (1.5×10^{-4} M) solution of human gamma globulin in controlled aggregation state and a 0.1 mg/ml (1.171×10^{-4} M) solution of paclitaxel in absolute ethanol are admixed in a 9:1 ratio and stirred until a clear solution is obtained.

10 The solution is lyophilised; the solid residue is redissolved in enough water to ensure a concentration of 16 % for human gamma globulin, obtaining a clear solution.

The binding is determined from UF filtrate and retentate fractions, showing 98% binding of paclitaxel to human gamma
15 globulin. This represents a 1: 0.71 ratio of human gamma globulin : paclitaxel.

In the above Examples II.1 through II.3 the quantity of paclitaxel was measured by HPLC following the method:

20 column MN Nucleosil C₁₈ 5µm 250x2 mm
mobile phase acetonitrile : water = 73 : 27
flow rate 0.30 ml/min
temperature ambient
detection at 273 nm
typical retention time 5.9 min; $k' = 2.93$

25 The substance was determined and found unchanged by LC/MS [see Rapid Communications in Mass Spectrometry VOL.11: p 1025-1032, 1997. and Rapid Communications in Mass Spectrometry, VOL. 9, p.495-502, 1995.]. The comparative results are shown in **Figures 6**: Figure 6A shows the mass
30 spectrum of the standard, Figure 6B shows the curve of the re-dissolved sample. Figure 6C shows the fragmentation of paclitaxel.

LC/MS parameters: ionisation: APCI + interface; nitrogen gas flow rate: 300 l/h; solvent: acetonitrile : buffer = 60 :
35 40, where the buffer is 10 mM ammonium formate pH 5.0 adjusted with 10% formic acid; flow rate: 0.300 ml/min.

Assay for the Determination of Paclitaxel:

A C-18 reverse phase HPLC method was applied for the quantitative determination of paclitaxel from different
40 solutions of Examples II. 1 through II. 27. The samples were injected into the HPLC system in ≥ 50 % ethanol solution, preventing any precipitation of the substance.

Binding

The binding of the substance to plasma proteins is determined after 15 minutes equilibration at $8 \pm 2^\circ \text{C}$.

The distribution of the substance is measurable after ultrafiltration through an appropriate membrane (cut-off must be $>$ than the M_w of the protein), determining the substance concentration in the ultrafiltrate fraction (representing the unbound) and in the prefiltered solution, releasing the bound part upon denaturation of the protein (representing the total). To denature the protein and release the bound fraction pre-cooled ($8 \pm 2^\circ \text{C}$) absolute ethanol is used in 1:1 ratio. The exact concentration values and amounts are calculated in consideration of the dilution factor.

Examples II.5 to II.21

The solution of human serum albumin in the concentration range of 20 % ($3.08 \times 10^{-3} \text{ M}$) to 0.02 % ($3.08 \times 10^{-6} \text{ M}$) is combined with the solution of paclitaxel in absolute ethanol in the concentration range from 20 mg/ml ($2.34 \times 10^{-2} \text{ M}$) to 0.01 mg/ml ($1.17 \times 10^{-5} \text{ M}$) obtaining always clear solutions. Details are presented in Table I. All measurements are performed three times and the calculated results are averaged.

TABLE I

Example	$[T]_T$ mM	[HSA] (mM)	$n(T_B)/n(\text{HSA})$	$n(T_B)/n(T_T) \times 100\%$
II.5	0.2342	2.410	0.093	97.4
II.6	0.2342	1.205	0.177	93.2
II.7	0.2342	0.602	0.346	91.0
II.8	0.2342	0.301	0.648	85.2
II.9	0.2342	0.121	1.545	81.2
II.10	0.2342	0.0602	3.125	82.1
II.11	0.2342	0.0241	5.662	59.5
II.12	0.2342	0.0121	4.948	26.0
II.13	0.2342	0.00602	5.823	15.3
II.14	0.2342	0.00241	10.419	11.0
II.15	0.2342	0.00121	14.367	7.6
II.16	0.2342	0.000602	12.370	3.3
II.17	4.6843	0.121	4.135	10.9
II.18	2.3421	0.121	8.401	44.2
II.19	1.1711	0.121	4.585	48.2
II.20	0.4648	0.121	2.864	75.3
II.21	0.1171	0.121	0.765	80.4

Legend:

- [T]_T total paclitaxel concentration after
addition to human serum albumin
- [HSA] concentration of human serum albumin
- 5 $n(T_B)/n(HSA)$ number of moles of paclitaxel bound per
mole of human serum albumin
- $n(T_B)/n(T_T) \times 100\%$ percentage of bound paclitaxel.

Variation of paclitaxel concentration (with 0.08 %
10 HSA, 10 % ethanol, 0.002 mg/ml paclitaxel) is shown on
Figure 7; variation of albumin concentration (with 0.004 -
16.0 % HSA, 20 % ethanol, 0.2 mg/ml paclitaxel) is shown
on **Figure 8**. variation of paclitaxel binding to HSA (with
0.8 % HSA, 10 % ethanol, 0.1 to 2.0 mg/ml paclitaxel) as a
15 function of pH at values of pH 4.0 to 8.5 is shown on
Figure 9. The signs on the graph correspond to the
following examples:

- Example II.18 -♦-♦-
- Example II.19 -O-O-
- 20 Example II.20 -x-x-
- Example II.15 -◇-◇-
- Example II.21 -▲-▲-

Example II.22

25 Similar methods as above in Examples II.2 to II.21
are used with animal serum albumin, immunoglobulin,
glycoproteins, interferons and interleukins.

Example II.23

30 Treatment of commercially available human serum albumin
or recombinant human serum albumin (in the following albumin)
to achieve the controlled aggregation state with the best
binding conditions of the molecule include removal of
stabilisers, such as sodium caprylate, N-acetyl-D,L-tryptophan
35 and other ionic components and salts.

a.) Ultrafiltration Method

Adjust the pH of the solution containing 10% albumin to
3.0 with hydrochloric acid and dilute to 5% protein content
with bi-distilled water. Concentrate the solution to 10% for
40 protein content using ultrafiltration (membrane cut off limit
30000 kD).

Dilute the solution back to 5% protein content with 1.0 mM hydrochloric acid. Concentrate the solution to 10% protein content using ultrafiltration (membrane cut off limit 30000 kD).

5 Repeat the procedure 12x, then adjust the pH to 6.9 with a 2.0 M aqueous sodium hydroxide solution and dilute the solution to 5% concentration for protein content with bi-distilled water. Concentrate the solution to 10% for protein content using ultrafiltration (membrane cut off limit 30000
10 kD) again.

Dilute the solution back to 5% for protein content with bi-distilled water. Concentrate the solution to 10% for protein content using ultrafiltration (membrane cut off limit 30000 kD). Repeat the procedure 10x, obtaining a pure protein
15 fraction, sufficiently free from other excipients. By that time, the conductivity of the ultrafiltrate is close to that of the bi-distilled water used for dilution. This protein is adequate for use to bind e.g. paclitaxel or cyclosporin.

b.)

20 Instead of ultrafiltration the use of dialysis gives similar results. The treatment requires about 48 hours.

Example II.24

The 0.8 % (1.203×10^{-4} M) solution of HSA and the 4.0
25 mg/ml (4.33×10^{-3} M) solution of amphotericin B (mw= 924.09) in DMF were mixed in a 9:1 ratio and stirred obtaining a clear solution.

The solution was lyophilised; the solid residue was redissolved using enough water to ensure that the concentra-
30 tion was 20% for HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 99.7% binding of amphotericin B to HSA. This represents a 1:4 ratio for HSA : amphotericin B.

Example II.25

35 The 0.8 % (1.203×10^{-4} M) solution of recombinant human serum albumin and the 40.0 mg/ml (4.33×10^{-2} M) solution of amphotericin B in DMF + HCl are mixed in a 9:1 ratio and stirred obtaining a clear solution.

40 The solution is lyophilised; the solid residue is redissolved in sufficient water to make the final concentration 20% for recombinant HSA, obtaining a clear

solution. The binding is determined from the UF filtrate and retentate fractions, showing 99.5% binding of amphotericin B to HSA. This represents a 1:40 ratio for recombinant HSA : amphotericin B.

5 Amphotericin B is measured by HPLC following the method below:

column MN Nucleosil C18 5 μ m 250x2 mm
mobile phase acetonitrile : buffer =1:1
(buffer : 0.2% formic acid pH adjusted to
10 4.0 with triethylamine)
flow rate 0.30 ml/min
temperature ambient
detection at 365 nm
typical retention time 5.3 min, $k' = 1.41$

15 The substance is determined and found unchanged using LC/MS. The comparative results are shown in **Figures 2**: Figure 2A shows the mass spectrum of the standard, Figure 2B shows the curve of the re-dissolved sample. Figure 2C shows the fragmentation of amphotericin B.

20 LC/MS parameters: ionisation: ESI + interface;
nitrogen gas flow rate: 300 l/h; solvent: 20 mM ammonium formate pH 4.0 adjusted with 10% formic acid; flow rate: 0.300 ml/min.

25 **Example II.26**

The 0.4 % (6.015×10^{-5} M) solution of HSA in controlled aggregation state and the 0.14 mg/ml (4.02×10^{-4} M) solution of camptothecin (mw= 348.36) in abs. ethanol were admixed in a 4:1 ratio and stirred to obtain a clear solution. The solution was lyophilised; the solid residue was redissolved in
30 enough water to ensure that the final concentration was 20% for HSA, thereby obtaining a clear solution. The binding was determined from the UF filtrate and retentate fractions, showing 98% binding of camptothecin to HSA. This represents a
35 1:5.34 ratio of HSA : camptothecin.

Example II.27

The 0.4 % (6.015×10^{-5} M) solution of recombinant HSA in controlled aggregation state and the 0.14 mg/ml (4.02×10^{-4} M)
40 solution of camptothecin in abs. ethanol were mixed in 4:1 ratio and stirred obtaining a clear solution.

The solution was lyophilised; the solid residue was redissolved in that much water as the final concentration was 20% for recombinant HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 98% binding of camptothecin to HSA. This represents a 1:5.34 ratio of recombinant HSA: camptothecin.

We measured the camptothecin by HPLC as follows:

column MN Nucleosil C₁₈ 5µm 250x2 mm

mobile phase acetonitrile : buffer=33:67

flow rate 0.33 ml/min

temperature ambient

detection at 356 nm.

typical retention time 6.9 min $k' = 2.45$

The substance was determined and found unchanged by

LC/MS [Cancer Research, VOL.56: p.3689-3694, 1996.]

Example II.29

The 4.0 % (6.015×10^{-4} M) solution of HSA in controlled aggregation state and the 8.0 mg/ml (3.39×10^{-2} M) solution of carbamazepin (mw 236.27) in abs. ethanol were admixed in 19:1 ratio and stirred obtaining a clear solution. The solution was lyophilised; the solid residue was redissolved in a sufficient amount of water to make the final concentration 20% for HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 98 % binding of carbamazepin to HSA. This represents a 1:2.8 ratio of HSA : carbamazepine.

The carbamazepin was measured by HPLC following the method below:

column MN Nucleosil C₁₈ 5µm 250x2 mm

mobile phase acetonitrile : buffer=1:1

(buffer: 0.2% formic acid pH adjusted to 7.0 with triethylamine)

flow rate 0.25 ml/min

temperature ambient

detection at 285 nm

typical retention time 5.3 min $k' = 1.12$

The substance was determined and found unchanged by

LC/MS [Eur. J. Clin. Chem Clin. Biochem, VOL. 35(10): p.755-759, 1997]. The comparative results are shown in **Figures 3:** Figure 3A shows the mass spectrum of the standard, Figure 3B

shows the curve of the re-dissolved sample. Figure 3C shows the fragmentation of carbamazepin.

LC/MS parameters: ionisation: ESI + interface; nitrogen gas flow rate: 300 l/h; solvent: 2 mM ammonium formate; flow rate: 0.250 ml/min.

Example II.30

The 4.0 % (6.015×10^{-4} M) solution of HSA in controlled aggregation state and the 1.0 mg/ml (8.33×10^{-4} M) solution of cyclosporine A (mw 1202.63) in absolute ethanol were mixed in 9:1 ratio and stirred obtaining a clear solution.

The solution was lyophilised; the solid residue was redissolved in a sufficient amount of water to make the final concentration 20% for HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 97 % binding of cyclosporine A to HSA.. This represents a 1:0.14 ratio for HSA : cyclosporine A.

Example II.31

The 2.0 % (3.008×10^{-4} M) solution of recombinant HSA in controlled aggregation state and the 1.0 mg/ml (8.33×10^{-4} M) solution of cyclosporine A in absolute ethanol were mixed in 9:1 ratio and stirred obtaining a clear solution.

The solution was lyophilised; the solid residue was redissolved in a sufficient amount of water to make the final concentration 20% for recombinant HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 98% binding of cyclosporine A to recombinant HSA. This represents a 1 : 0.29 ratio for recombinant HSA : cyclosporine A.

Example II.32

The 2.25 % (1.50×10^{-4} M) solution of human gamma globulin and the 1.0 mg/ml (8.33×10^{-4} M) solution of cyclosporine A in absolute ethanol were mixed in a 9:1 ratio and stirred obtaining a clear solution.

The solution is lyophilised; the solid residue is redissolved in enough water to give a concentration of 16% for human gamma globulin, thereby obtaining a clear solution. The binding is determined from the UF filtrate and retentate fractions, showing 98 % binding of cyclosporine A to human

gamma globulin.. This represents a 1 : 0.56 ratio for human gamma globulin : cyclosporine A.

The cyclosporine A was measured by HPLC following the method below:

5 column MN Nucleosil C18 5µm 250x2 mm
 mobile phase acetonitrile:water : methanol :
phosphoric acid = 700:260:40:0.05
 flow rate 0.350 ml/min
 temperature 80 °C thermostat
10 detection at 205 nm
 typical retention time 7.5 min k' = 2.95

The substance was determined and found unchanged by LC/MS [1], as the results show. The comparative results are shown in **Figures 4**: Figure 4A shows the mass spectrum of the
15 standard, Figure 4B shows the curve of the re-dissolved sample. Figure 4C shows the fragmentation of cyclosporine A.

LC/MS parameters: ionisation: ESI + interface, nitrogen gas flow rate: 300 l/h; solvent: acetonitrile/ water = 60 / 40; solvent flow rate: 0.350 ml/min.

20

Example II.33

The 0.4 % (6.015×10^{-5} M) solution of HSA and the 2.0 mg/ml (1.12×10^{-2} M) solution of propofol (mw 178.27) in absolute ethanol were mixed in 9:1 ratio and stirred obtaining
25 a clear solution.

The solution was lyophilised; the solid residue was redissolved in a sufficient amount of water to make the final concentration 20% for HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate
30 fractions, showing 99% binding of propofol to HSA. This represents a 1:18.3 ratio of HSA : propofol.

Example II.34

The 0.4 % (6.015×10^{-5} M) solution of recombinant HSA
35 and the 2.0 mg/ml (1.12×10^{-2} M) solution of propofol in absolute ethanol were mixed in 9:1 ratio and stirred obtaining a clear solution.

The solution is lyophilised; the solid residue was redissolved in a sufficient amount of water to make the final
40 concentration 20% for recombinant HSA, obtaining a clear solution. The binding was determined from UF filtrate and retentate fractions, showing 99% binding of propofol to

recombinant HSA. This represents a 1 : 18.3 ratio of recombinant HSA : propofol.

Propofol was measured by HPLC as follows:

column MN Nucleosil C₁₈ 5 μ m 250x2 mm
mobile phase acetonitrile : water=73:27
flow rate 0.30 ml/min
temperature ambient
detection at 273 nm
typical retention time 6.1 min $k' = 1.77$

The substance was determined and found unchanged by LC/MS [J.of Chromatography B, 669: p. 358 - 365, 1995]. The comparative results are shown in **Figures 5**: Figure 5A shows the mass spectrum of the standard, Figure 5B shows the curve of the re-dissolved sample. Figure 5C shows the fragmentation of propofol.

LC/MS parameters:

ionisation: APCI + interface; nitrogen flow rate: 300 l/h;
solvent: acetonitrile / water = 73 / 23; flow rate: 0.300 ml/min.

Example II.35

9.0 ml of a 0.8% (1.213×10^{-4} M) solution of HSA and 1.0 ml of a 4.0 mg/ml (4.33×10^{-3} M) solution of amphotericin B in dimethyl formamide were mixed to give a clear solution. This solution was dialyzed against 2.0 liter of water (WFI) at 4 °C for period of 20 hours protected from light.

Using the determination method of Example II.24 the binding was found to be 99.6% representing a 1 : 3.5 ratio for HSA: amphotericin B.

On repeating the dialysis procedure five times the concentration of DMF in the solution was reduced below its detection limit (2×10^{-9} M).

III. DOSAGE FORMS

Examples III.1 to III.6

Following the procedure for the preparation with lyophilisation as described above an appropriate pharmaceutical formulation is obtained. Re-dissolving the solid in adequate volume of WFI so as to reach the concentration of 20% for HSA the solution arrives to a concentration suitable for therapeutic application as summarised below for some active substances:

Example	name.	conc. mg/ml
III.1	amphotericin B	11.09
III.2	camptothecin	6.8
III.3	carbamazepine	1.98
III.4	cyclosporin A	0.50
III.5	paclitaxel	1.0
III.6	propofol	10.0

The above dosage forms can be further finished in vials for injectables and infusions.

IV. BIOLOGICAL EXAMPLES

Studies on Biological Equivalence

Biological equivalence was determined comparing the new formulations according to the invention with known formulations used in therapy containing the same active substance with poor water solubility. Such known formulations were prepared in polyoxyethylated castor oil (Cremophor EL) and absolute ethanol.

Materials used:

Paclitaxel dissolved in a mixture of polyoxyethylated castor oil (Cremophor EL): absolute ethanol = 1:1, was compared with the aqueous solution of paclitaxel/HSA of the invention, prepared according to Example II.2.

Example IV.1 In vitro studies

Comparative studies were carried out in vitro to determine the antiproliferative and cytotoxic activity on human tumour cell lines. The Cremophor EL/absolute ethanol and HSA formulation of paclitaxel was compared on K562 human myeloid leukaemia, MCF-7 and MDA-231 breast and OVCAR-5

ovarian carcinoma cell lines [Anticancer Research, Vol. 16: p.2469-2478, 1996.]

Method:

Colony growth inhibition assay: Monolayer cultures of the cell lines were treated with eight different concentrations of the drug in the two above formulations plus in DMSO/saline solution as a reference. The cultures were incubated for 24, 48, 72, 96 and 120 hours respectively. The colonies were stained with crystal violet and the survival of treated cells was calculated as percentage of colonies formed by untreated cells. **Tables II A** through **IV B** show the results obtained on the different cell lines. In each study the survival of treated cells is shown, calculated as percentage of colonies formed by untreated cells. All values are the average of three experiments.

TABLE II A

cell line: MCF7 breast carcinoma

formulation: paclitaxel/Cremophor EL & absolute ethanol

Ptx cc[uM] \ t[h]	24h	48h	72h	96h	120h
0.005	92*	86	76	42	30
0.01	90	81	72	33	26
0.02	86	71	67	29	23
0.025	84	64	60	24	18
0.05	82	60	52	23	16
0.1	80	57	38	18	15
1.0	68	46	28	15	6.5
10.0	62	33	21	10	4.6

20

TABLE II B

cell line: MCF7 breast carcinoma

formulation: paclitaxel/HSA

Ptx cc[uM] \ time [h]	24h	48h	72h	96h	120h
0.005	91	84	75	41	27
0.01	88	81	69	35	23
0.02	84	76	64	31	20
0.025	80	70	59	28	16
0.05	77	66	53	25	12
0.1	75	59	46	20	9.5
1.0	67	42	30	17	6.2
10.0	58	31	21	9.0	3.0

TABLE III A

cell line: MDA-231 breast carcinoma

formulation: paclitaxel/Cremophor EL & absolute ethanol

Ptx cc[μ M] \ time [h]	24h	48h	72h	96h	120h
0.005	97	89	80	47	34
0.01	94	87	75	41	30
0.02	89	82	69	37	28
0.025	86	76	65	34	23
0.05	84	72	59	29	21
0.1	83	66	53	26	18
1.0	73	49	34	21	9.5
10.0	65	37	24	14	8.2

5

TABLE III B

cell line: MDA-231 breast carcinoma

formulation: paclitaxel/HSA

Ptx cc[μ M] \ time [h]	24h	48h	72h	96h	120h
0.005	92	78	51	30	10
0.01	86	65	38	24	8.3
0.02	75	51	33	22	7.0
0.025	64	47	28	19	6.4
0.05	60	42	26	18	5.3
0.1	55	36	24	16	4.0
1.0	49	33	22	15	3.2
10.0	45	26	20	10	2.6

10

TABLE IV A

cell line: K562 human myeloid leukaemia

formulation: paclitaxel/Cremophor EL & absolute ethanol

Ptx cc[μ M] \ time [h]	24h	48h	72h	96h	120h
0.005	88	59	30	21	10
0.01	79	40	21	15	8.7
0.02	66	31	19	12	7.2
0.025	62	29	17	10	6.0
0.05	56	25	14	9.4	5.4
0.1	51	23	12	7.7	4.6
1.0	47	20	10.5	6.0	3.0
10.0	39	16	9.5	4.2	2.0

TABLE IV B

cell line: K562 human myeloid leukaemia sample: paclitaxel/HSA

Ptx cc[μ M] \ time [h]	24h	48h	72h	96h	120h
0.005	89	53	31	18	5.4
0.01	75	40	22	11	4.7
0.02	69	32	18	9.0	4.0
0.025	65	30	14	7.6	3.5
0.05	58	25	11	7.0	3.0
0.1	53	21	9.5	5.6	2.4
1.0	47	18	8.0	5.0	1.7
10.0	41	16	7.1	4.7	1.0

Example IV.2: In Vivo Pharmacokinetic Test

From the therapeutic point of view, the bio-equivalence can be considered, demonstrating equal pharmacokinetic characteristics such as AUC (area under the curve), elimination constants, plasma half life after the administration of the same dose to the same species. Such experiment was done on rats for the two formulations as in Example IV.1. [Semin Oncol, VOL. 21 (5 Suppl. 8): p.53-62, 1994.].

AUC means the area under the curve on a plasma concentration versus time diagram. It can be generated measuring the plasma concentration of the compound administered at different points of time.

15 Pharmacokinetic study on rats:

Method: The dose of 2.5 mg/kg paclitaxel was administered in 1.0 ml volume i.v.. bolus to CR. (Wi) BR rats (body weight between 380 and 420 grams), and a 1.0 ml blood sample was drawn into a heparinised test tube from three
20 animals at each point of time as indicated below:

[illegible]

The plasma fraction was separated by quick centrifugation at +5°C and kept frozen at -70°C until processed for analytical measurement.

5 Sample preparation: The frozen plasma samples were warmed up to +8 °C, centrifuged for 5 min at 5000 RPM. 0.300 - 0.500 ml of the clear plasma solution was taken out and loaded onto an Oasis HLB 1 cc SPE (Solid Phase) Extraction cartridge. Before the plasma sample was loaded the cartridge was rinsed
10 with 1 ml of methanol, followed by 1 ml water for pre-conditioning. The paclitaxel content absorbed onto the SPE cartridge, while the rest of the sample components were rinsed out with 1 ml of water and 1 ml of 30% acetonitrile/water solution. The cartridge was blown dry by air. Paclitaxel was
15 eluted from the SPE cartridge with 1 ml absolute ethanol. The sample was evaporated to dryness with nitrogen, stored at (-20°C) for analysis. The residue was dissolved in 0.200 ml absolute ethanol and injected for HPLC analysis.

20 The HPLC conditions were the same as applied for substance identification.

Results:

The points obtained were the average of three measured from the samples of three individual animals. As a result, the difference between the two curves obtained from the
25 pharmacokinetic study for the equal dose of the two different formulations, remained within the deviation of the individual samples. The same curve takes shape plotting all the individual data, indicating no or minor difference in pharmacokinetic characteristics of the two formulations.

30

Example IV.3

In vivo evaluation of antiproliferative and cytotoxic activity investigations show that the new formulations shows a positive effect against human tumour xenografts CH1 and CH1_{tax}
35 in nude mice.

Example IV.4

Hypersensitivity Tests

About 45% of the patients treated with paclitaxel
40 expressed hypersensitivity reactions. These side effects were proven related to one excipient of the formulation, Cremophor EL, as observed with other pharmaceutical products containing

the same component. This hypersensitivity reaction is determined as anaphylactic toxicity expressed through induction of histamine release by Cremophor EL.

Our study was performed on CRL (WI) BR male rats weighing 130 - 150 g [14]. The administered dose was calculated around 7.0 mg/kg for paclitaxel, given i.v. in 1.0 ml of total volume. A group for each time point and dose contained 5 animals. The blood samples were collected into heparin containing tubes after 2, 5 and 10 minutes of treatment. The plasma was separated by quick centrifugation. The samples were stored at -70°C .

The histamine content of the sample was C¹⁴-methylated by specific histamine-N-methyl-transferase enzyme. The histamine level was determined in the plasma samples measuring the C¹⁴ radioactivity in the samples.

The data obtained indicated that Cremophor EL and the containing formulation have substantial histamine release induction, while HSA and the HSA containing formulation and paclitaxel itself do not show any such effect.

Example IV.5

The same phenomenon as in Example IV.4 was found using *in vitro* human experiments from human blood samples based on the quantitative assay of chromatin activation of blood lymphocytes [Method: Analytical and Quantitative Cytology and Histology, VOL. 8: p.1, 1986.].

CLAIMS

- 5 1. A water-soluble product or pharmaceutical
formulation in solid or liquid form mainly for parenteral
use containing
- a) a therapeutically active compound having low aqueous
solubility and a substantial binding affinity to plasma
10 proteins (in the following "active substance") and
- b) a plasma protein fraction in controlled aggregation
state
whereby the said active substance and the said protein fraction
are bound to each other by way of non-covalent bonds and
- 15 c) optionally further pharmaceutically acceptable and
mainly parenterally acceptable formulation additive(s) - such
as water, stabilizer(s), protein aggregation controller(s).
2. A water-soluble product or human pharmaceutical
20 formulation according to claim 1 mainly for parenteral use
containing a therapeutically active substance having low
aqueous solubility and a human plasma protein fraction in
controlled aggregation state.
- 25 3. A water-soluble product or veterinary pharmaceutical
formulation according to claim 1 mainly for parenteral use
containing a therapeutically active substance having low
aqueous solubility and an animal plasma protein fraction in
controlled aggregation state.
- 30 4. A product or pharmaceutical formulation according to
any of claims 1 to 3 containing a component of the natural -
human or animal - plasma such as serum albumin, an
immunoglobulin, glycoprotein, interferon and/or interleukin or
35 a recombinant of said plasma component.
5. A product or pharmaceutical formulation according to
any of claims 1 to 4 for human administration, mainly for
parenteral use, containing a component of the natural - human
40 plasma such as serum albumin, an immunoglobulin, glycoprotein,
interferon and/or interleukin or a recombinant of said plasma

component.

6. A product or pharmaceutical formulation according to any of claims 1 to 3 containing as the water-insoluble active substance a cytostatic such as a taxonoid, an antibiotic, vitamin, antiinflammatory, analgesic, antiviral, anticonvulsant, immunosuppressant, antiepileptic, anxiolytic, hypnotic, antifungal agent, anticoagulant, lipid peroxidase inhibitor, coronary vasodilator, antiarrhythmic agent, cardiotonic, uricosuric, antithrombotic, steroid hormone (progestogen, androgen, testosterone) and/or photosensitizer.

7. A product or pharmaceutical formulation according to any of claims 1 to 6 containing at least one of the following active substances: amphotericin B, an adriamycin analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyridamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid, warfarin.

8. A product or pharmaceutical formulation according to any of claims 1 to 7 containing a taxonoid of the general formula I - in the formula

R^1 represents tert. butyl-oxy-carboxylic acid amide or benzoyl amide

R^2 represents hydrogen or any acyl group preferably acetyl.

9. A product or pharmaceutical formulation according to any of claims 1 to 7 containing paclitaxel and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably in the molar ratio of 1 : 0.1 to 1 : 50.

10. A product or pharmaceutical formulation according to any of claims 1 to 9 containing azathioprine and human serum albumin, immunoglobulin, glycoprotein, interferon and/or

interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

5 11. A formulation according to any of claims 1 to 8 containing camptothecin and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1
10 to 1 : 50.

 12. A formulation according to any of claims 1 to 8 containing gemfibrozil and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other
15 natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

 13. A product or pharmaceutical formulation according to
20 any of claims 1 to 8 containing miconazole and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

25 14. A product or pharmaceutical formulation according to any of claims 1 to 8 containing propofol and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma
30 protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

 15. A product or pharmaceutical formulation according to any of claims 1 to 8 containing tamoxifen and human serum
35 albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

40 16. A product or pharmaceutical formulation according to any of claims 1 to 8 containing ritonavir and human serum albumin, immunoglobulin, glycoprotein, interferon and/or

interleukin or some other natural or recombinant human plasma protein fraction the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

5 17. A pharmaceutical formulation according to any of claims 1 to 8 containing tacrolimus and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1
10 to 1 : 50.

 18. A pharmaceutical formulation according to any of claims 1 to 8 containing tirilazad and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or
15 some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

 19. A pharmaceutical formulation according to any of
20 claims 1 to 8 containing trioxsalen and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

25 20. A pharmaceutical formulation according to any of claims 1 to 19 having a solid state or having the form of an aqueous solution.

 21. A pharmaceutical formulation according to any of
30 claims 1 to 20 containing as additive an agent stabilizing the solution and/or the protein.

 22. A pharmaceutical formulation according to claim 21 containing as solution and/or protein stabilizing agent any of the following: sodium chloride, a buffer, an alcohol such as
35 glycerol and/or a water-soluble sugar derivative preferably mannitol, sorbitol, dulcitol.

 23. Process for the preparation of a product or
40 pharmaceutical formulation according to any of claims 1 to 22 or a new product according to claims 30 to 37
characterized b y

a) dissolving the therapeutically active compound having low aqueous solubility and a substantial binding affinity to plasma proteins ("active substance") in a water-miscible, pharmaceutically acceptable organic solvent,

5 b) combining said solution with the aqueous solution of a plasma protein fraction in controlled aggregation state and optionally

c) a further pharmaceutically acceptable auxiliary additive - such as a protein aggregation controller and/or a
10 stabilizer -

whereby a true solution is obtained containing the said active substance and the said protein fraction bound together by way of non-covalent bonds;

d) removing the organic solvent preferably by
15 ultrafiltering, dialysing, diafiltrating and/or lyophilising the solution or its concentrate or by combination of these treatments

whereby a homogeneous, water-soluble liquid or solid pharmaceutical product is obtained containing the active
20 substance and the plasma protein fraction;

e) optionally dissolving the solid product in water or diluting the liquid product with water whereby a clear, liquid composition is obtained which is suitable for
therapeutical administration and

25 f) optionally finishing this product into a parenteral formulation (dosage form) for direct use.

25. Process for the preparation of a product or pharmaceutical formulation according to any of claims 1 to 24
30 or 30 to 37 characterized by

a) dissolving the therapeutically active compound in a water-miscible, pharmaceutically acceptable organic solvent,

b) combining said solution with the aqueous solution of the selected plasma protein fraction in controlled
35 aggregation state, said solution containing optionally

c) a further pharmaceutically acceptable auxiliary additive - such as a protein aggregation controller and/or a stabilizer -

whereby a true solution is obtained containing the
40 said active substance and the said protein fraction bound together by way of non-covalent bonds;

d) removing the organic solvent and lyophilising the solution or its concentrate.

26. A process according to step a) of any of claims 23 to 25 characterized by using to dissolve the active substance a solvent having the following properties:

a) it is capable to completely dissolve the active substance in its mixture with water and

b) its mixture with <50% of water does not denaturalize the protein employed.

27. A process according to claim 26 characterized by using as the solvent any of the group an aliphatic C(2-4) monoalcohol or polyalcohol, 70 - 100% ethanol, dimethyl formamide, methyl formamide.

28. A process according to step a) of claim 23 to 27 characterized by using as protein aggregation controller or stabilizer and/or solution stabilizing auxiliary additive any of the following agents: water, sodium chloride, a buffer, a poly- alcohol such as glycerol and/or a water-soluble sugar derivative preferably mannitol, sorbitol and/or dulcitol.

29. A process according to step a) of any of claims 23 to 28 characterized by using paclitaxel and a component of the natural plasma such as serum albumin, an immunoglobulin, glycoprotein, interferon and/or interleukin or a recombinant of the same.

30. A homogeneous, solid, water-soluble product consisting of at least one active substance of the group amphotericin B, an adriamycin analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyrindamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid and/or warfarin

and also consisting of at least one protein of the group human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction

- 5 where the said active substance and the said protein fraction are bound to each other by way of non-covalent bonds and wherein the molar ratio of the said active substance and the said protein fraction is within the range of 1 : 0.05 to 1 : 100, preferably of 1 : 0.1 to 1 : 50.

10

31. A homogeneous, solid, water-soluble product according to claim 30 consisting of a taxonoide of the general formula I - in the formula

- 15 R^1 represents tert. butyl-oxy-carboxylic acid amide or benzoyl amide,
 R^2 represents hydrogen or any acyl group preferably acetyl -
and of a plasma protein fraction.

20

32. A homogeneous, solid, water-soluble product according to claim 30 consisting of paclitaxel and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

- 25 33. A homogeneous, solid, water-soluble product according to claim 30 consisting of amphotericin B and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

- 30 34. A homogeneous, solid, water-soluble product according to claim 30 consisting of camptothecin and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

- 35 35. A homogeneous, solid, water-soluble product according to claim 28 consisting of carbamazepin and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

- 40 36. A homogeneous, solid, water-soluble product according to claim 28 consisting of cyclosporin A and human serum albumin, recombinant human plasma albumin and/or γ -

globulin.

37. A homogeneous, solid, water-soluble product according to claim 30 consisting of propofol and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

38. Method of treatment of human or veterinary patients with a water-insoluble therapeutically active substance having substantial plasma protein affinity characterized by administering to a patient in need of a treatment with said active substance an effective dose of the product or pharmaceutical formulation according to or prepared according to any of claims 1 to 37.

39. Method of parenteral treatment of human or veterinary patients with a water-insoluble therapeutically active substance having substantial affinity for binding to plasma protein characterized by parenterally administering to a patient in need of a treatment with said active substance an effective dose of the following products preferably using the following dose ranges respectively (calculated on the active substance): paclitaxel/albumin 70 - 280 mg/treatment; propofol/albumin 6 - 10 mg/kg/hour; camptothecin/albumin, gemfibrozil/albumin, cyclosporin A/albumin 3 - 5 mg/kg/day; amphotericin B/ albumin up to 1.5 mg/kg/day, whereby the same dose ranges are used for compounds containig the recombinant proteins respectively.

40. Method for parenteral delivery in therapeutic use of pharmaceutically active ingredients with poor solubility in water and substantial affinity for binding to plasma proteins characterized by administering to a patient in need of a treatment with said active substance an effective dose of the composition according to or prepared according to any of claims 1 to 19.

41. A product or method substantially as described in any of the examples.

AMENDED CLAIMS

[received by the International Bureau on 05 January 1999 (05.01.99);
original claims 1,2-5,23,25,30 and 38-40 replaced by amended claims 1,3-6,24,25,30 and 38-40;
new claim 2 added; original claims 6-22 renumbered 7-23; remaining claims unchanged (9 pages)]

1. A water-soluble product or pharmaceutical
formulation in solid or liquid form and their organic solvent-
free true aqueous solutions mainly for parenteral use
containing

a) a therapeutically active compound having low aqueous
solubility ($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) and a
substantial binding affinity to plasma proteins (in the
following "active substance") in an interlinked state with

b) a plasma protein fraction in controlled aggregation
state

whereby the said active substance and the said protein fraction
are bound to each other by way of non-covalent bonds and

optionally further containing

c) pharmaceutically acceptable and mainly parenterally
acceptable watersoluble formulation additive(s) - such as
water, stabilizer(s), protein aggregation controller(s).

2. A water-soluble product or pharmaceutical
formulation in solid or liquid form and their organic solvent-
free aqueous solutions according to claim 1 wherein the molar
ratio of the active ingredient : protein is within the range of
1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50

3. A water-soluble product or human pharmaceutical
formulation according to claim 1 or 2 containing a human plasma
protein fraction in controlled aggregation state.

4. A water-soluble product or veterinary pharmaceutical
formulation according to claim 1 or 2 containing an animal
plasma protein fraction in controlled aggregation state.

5. A product or pharmaceutical formulation according to
any of claims 1 to 4 containing as the plasma protein fraction
a component of the natural plasma such as serum albumin or a
recombinant of said plasma component.

6. A product or pharmaceutical formulation according to
any of claims 1 to 5 containing as the plasma protein fraction

a natural immunoglobulin, glycoprotein, interferon and/or interleukin or a recombinant of said plasma component.

5 7. A product or pharmaceutical formulation according to any of claims 1 to 6 containing as the water-insoluble active substance a cytostatic such as a taxonoid, an antibiotic, vitamin, antiinflammatory, analgesic, antiviral, anticonvulsant, immunosuppressant, antiepileptic, anxiolytic, hypnotic, antifungal agent, anticoagulant, lipid peroxidase
10 inhibitor, coronary vasodilator, antiarrhythmic agent, cardiotonic, uricosuric, antithrombotic, steroid hormone (progestogen, androgen, testogen) and/or photosensitizer.

15 8. A product or pharmaceutical formulation according to any of claims 1 to 6 containing at least one of the following active substances: amphotericin B, an adriamycin analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, dipyridamole, disopyramide,
20 flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid, warfarin.

25 9. A product or pharmaceutical formulation according to any of claims 1 to 7 containing a taxonoid of the general formula I - in the formula

30 R^1 represents tert. butyl-oxy-carboxylic acid amide or benzoyl amide,

R^2 represents hydrogen or an acyl group preferably acetyl.

35 10. A product or pharmaceutical formulation according to any of claims 1 to 7 containing paclitaxel and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably in the molar ratio of 1 : 0.1 to 1 : 50.

40 11. A product or pharmaceutical formulation according to any of claims 1 to 9 containing azathioprine and human serum

albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

5

12. A formulation according to any of claims 1 to 8 containing camptothecin and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

10

13. A formulation according to any of claims 1 to 8 containing gemfibrozil and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

15

14. A product or pharmaceutical formulation according to any of claims 1 to 8 containing miconazole and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

20

25

15. A product or pharmaceutical formulation according to any of claims 1 to 8 containing propofol and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

30

16. A product or pharmaceutical formulation according to any of claims 1 to 8 containing tamoxifen and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

35

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17. A product or pharmaceutical formulation according to any of claims 1 to 8 containing ritonavir and human serum

albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

5

18. A pharmaceutical formulation according to any of claims 1 to 8 containing tacrolimus and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

10

19. A pharmaceutical formulation according to any of claims 1 to 8 containing tirilazad and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

15

20. A pharmaceutical formulation according to any of claims 1 to 8 containing trioxsalen and human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction in the molar ratio of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50.

20

25

21. A pharmaceutical formulation according to any of claims 1 to 20 having a solid state or having the form of an aqueous solution.

30

22. A pharmaceutical formulation according to any of claims 1 to 20 containing as additive an agent stabilizing the solution and/or the protein.

35

23. A pharmaceutical formulation according to claim 22 containing as solution- and/or protein-stabilizing agent any of the following: sodium chloride, a buffer, an alcohol such as glycerol and/or a water-soluble sugar derivative preferably mannitol, sorbitol, dulcitol.

40

24. Process for the preparation of a water-soluble product or pharmaceutical formulation in solid or liquid form

according to any of claims 1 to 23 or a new product according to claims 30 to 37 and their organic solvent-free true aqueous solutions characterized by preparing a true aqueous solutions by way of

5 a) dissolving the therapeutically active compound having low aqueous solubility ($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) and a substantial binding affinity to plasma proteins ("active substance") in a water-miscible, pharmaceutically acceptable organic solvent,

10 b) combining said solution with the aqueous solution of the plasma protein fraction in controlled aggregation state

c) and optionally with a further pharmaceutically acceptable water-soluble auxiliary additive - such as a protein aggregation controller and/or a stabilizer -

whereby a true solution is obtained containing the said active substance and the said protein fraction bound together by way of non-covalent bonds;

20 d) removing the organic solvent and optionally the water preferably by ultrafiltering, dialysing, diafiltrating and/or lyophilising the solution or its concentrate or by combination of these treatments

whereby a homogeneous, water-soluble liquid or solid pharmaceutical product is obtained containing the active substance interlinked with the plasma protein fraction;

25 e) optionally dissolving the solid product in water or diluting the liquid product with water whereby a clear, true aqueous solution, free of any organic solvent is obtained which is suitable for therapeutical administration and

30 f) optionally finishing this product into a parenteral formulation (dosage form) for direct use.

35 25. Process for the preparation of a water-soluble product or pharmaceutical formulation in solid or liquid form according to any of claims 1 to 23 or a new product according to claims 30 to 37 and their organic solvent-free true aqueous solutions characterized by

40 a) dissolving the therapeutically active compound in a water-miscible, pharmaceutically acceptable organic solvent,

b) combining said solution with the aqueous solution of the selected plasma protein fraction in controlled aggregation state,

5 c) said solution containing optionally a further pharmaceutically acceptable auxiliary additive - such as a protein aggregation controller and/or a stabilizer -

whereby a true solution is obtained containing the said active substance and the said protein fraction bound together
10 by way of non-covalent bonds;

d) removing the organic solvent and lyophilising the solution or its concentrate.

26. A process according to step a) of any of claims 23
15 to 25 characterized by using to dissolve the active substance a solvent having the following properties:

a) it is capable to completely dissolve the active substance in its mixture with water and

b) its mixture with <50% of water does not denaturalize
20 the protein employed.

27. A process according to claim 26 characterized by using as the solvent any of the group consisting of an aliphatic C₍₂₋₄₎ monoalcohol or polyalcohol,
25 70 - 100% ethanol, dimethyl formamide, methyl formamide.

28. A process according to step a) of claim 23 to 27 characterized by using as protein aggregation controller or stabilizer and/or solution stabilizing auxiliary
30 additive any of the following agents: water, sodium chloride, a buffer, a poly- alcohol such as glycerol and/or a water-soluble sugar derivative preferably mannitol, sorbitol and/or dulcitol.

35 29. A process according to step a) of any of claims 23 to 28 characterized by using paclitaxel and a component of the natural plasma such as serum albumin, an immunoglobulin, glycoprotein, interferon and/or interleukin or a recombinant of the same.

40 30. A homogeneous, solid, water-soluble product consisting of at least one active substance having low aqueous

solubility ($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) of the group amphotericin B, an adriamycin analogue, apazone, azathioprine, bromazepam, camptothecin, carbamazepine, clonazepam, cyclosporine A, diazepam, dicumarol, digitoxine, 5 dipyridamole, disopyramide, flunitrazepam, gemfibrozil, ketochlorin, ketoconazole, miconazole, niflumic acid, oxazepam, phenobarbital, phenytoin, progesterone, propofol, ritonavir, sulfinpyrazone, suprofen, tacrolimus, tamoxifen, taxonoid, testosterone, tirilazad, trioxsalen, valproic acid and/or 10 warfarin

and also consisting of at least one protein of the group human serum albumin, immunoglobulin, glycoprotein, interferon and/or interleukin or some other natural or recombinant human plasma protein fraction

15 where the said active substance and the said protein fraction are bound to each other by way of non-covalent bonds and wherein the molar ratio of the said active substance and the said protein fraction is within the range of 1 : 0.05 to 1 : 100, preferably of 1 : 0.1 to 1 : 50.

20 31. A homogeneous, solid, water-soluble product according to claim 30 consisting of a taxonoide of the general formula I - in the formula

25 R^1 represents tert. butyl-oxy-carboxylic acid amide or benzoyl amide,

R^2 represents hydrogen or any acyl group preferably acetyl -

and of a plasma protein fraction.

30 32. A homogeneous, solid, water-soluble product according to claim 30 consisting of paclitaxel and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

35 33. A homogeneous, solid, water-soluble product according to claim 30 consisting of amphotericin B and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

40 34. A homogeneous, solid, water-soluble product according to claim 30 consisting of camptothecin and human serum albumin, recombinant human plasma albumin and/or γ -

globulin.

35. A homogeneous, solid, water-soluble product according to claim 28 consisting of carbamazepin and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

36. A homogeneous, solid, water-soluble product according to claim 28 consisting of cyclosporin A and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

37. A homogeneous, solid, water-soluble product according to claim 30 consisting of propofol and human serum albumin, recombinant human plasma albumin and/or γ -globulin.

38. Method of treatment of human or veterinary patients with a therapeutically active substance having low aqueous solubility ($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) and having substantial plasma protein affinity characterized by administering to a patient in need of a treatment with said active substance an effective dose of the product or pharmaceutical formulation according to or prepared according to any of claims 1 to 37.

39. Method of treatment of human or veterinary patients with a therapeutically active substance having low aqueous solubility ($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) and having substantial plasma protein affinity characterized by parenterally administering to a patient in need of a treatment with said active substance an effective dose of the following products preferably using the following dose ranges respectively (calculated on the active substance): paclitaxel/albumin 70 - 280 mg/treatment; propofol/albumin 6 - 10 mg/kg/hour; camptothecin/albumin, gemfibrozil/albumin, cyclosporin A/ albumin 3 - 5 mg/kg/day; amphotericin B/ albumin up to 1.5 mg/kg/day, whereby the same dose ranges are used for compounds containig the recombinant proteins respectively.

40. Method for parenteral delivery in therapeutic use of pharmaceutically active ingredients with poor solubility

($<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit) and substantial affinity for binding to plasma proteins characterized by administering to a patient in need of a treatment with said active substance an effective dose of the composition
5 according to or prepared according to any of claims 1 to 29.

41. A product or method substantially as described in any of the examples.

STATEMENT UNDER ARTICLE 19

5

1. Original Claims 8 to 23, 29, 31, 32, 34, 36, 37, 39 to 41
were found to be new by the Search Report. These claims -
with the exception of claim 40 - remained unchanged but to
the correction of reference numbers where adequate. (They
had to be partly renumbered because a new Claim 2 was in-
serted.)
2. Amended composition claim 1, new claim 2, amended process
claim 24, compound claim 30, and method of treatment
claims 39 to 40 are now amended by insertion of basically
three limitations:
 - a) It is now emphasized that pharmaceutical formulations
to be used mainly for parenteral use **are organic sol-
vent-free true aqueous solutions.**
This excludes pharmaceuticals for parenteral use
which are
 - i) suspensions or emulsions (such as those dis-
closed in EPA A1 326618 prepared by way of high
speed stirring of a two or three phase system);
 - ii) solutions and emulsions containing organic sol-
vents such as ethanol (such as those disclosed
in DE A1 3702105);
 - b) The low aqueous solubility is now indicated to be
 $<1.10^{-4}$ M/Lit, $<1.10^{-5}$ M/Lit, $<1.10^{-6}$ M/Lit.
This limitation excludes the use of active ingredi-
ents which have a higher aqueous solubility of about
 ≤ 0.1 mg/ml such as disclosed in the Patent Abstract of
JP 58216126 mentioned in the Search report. Some of

such more soluble active ingredients were disclosed to form aqueous solutions by simple dissolution in water - which solutions in turn could be solubilized by addition of HSA as solubilizing agent.

5 According to the present invention compounds having solubilities several magnitudes less can be transformed into water-soluble products - while the method disclosed in the mentioned prior art can not be used for products having solubilities as now claimed.

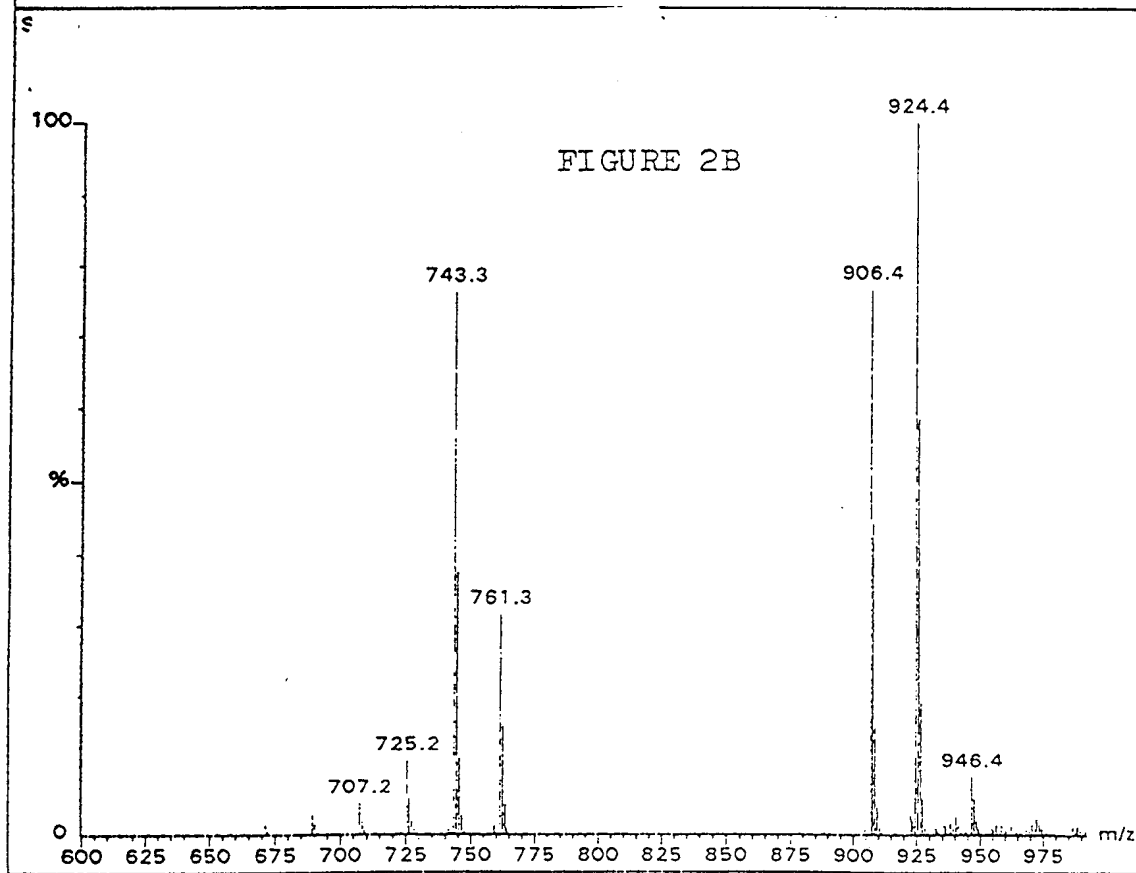
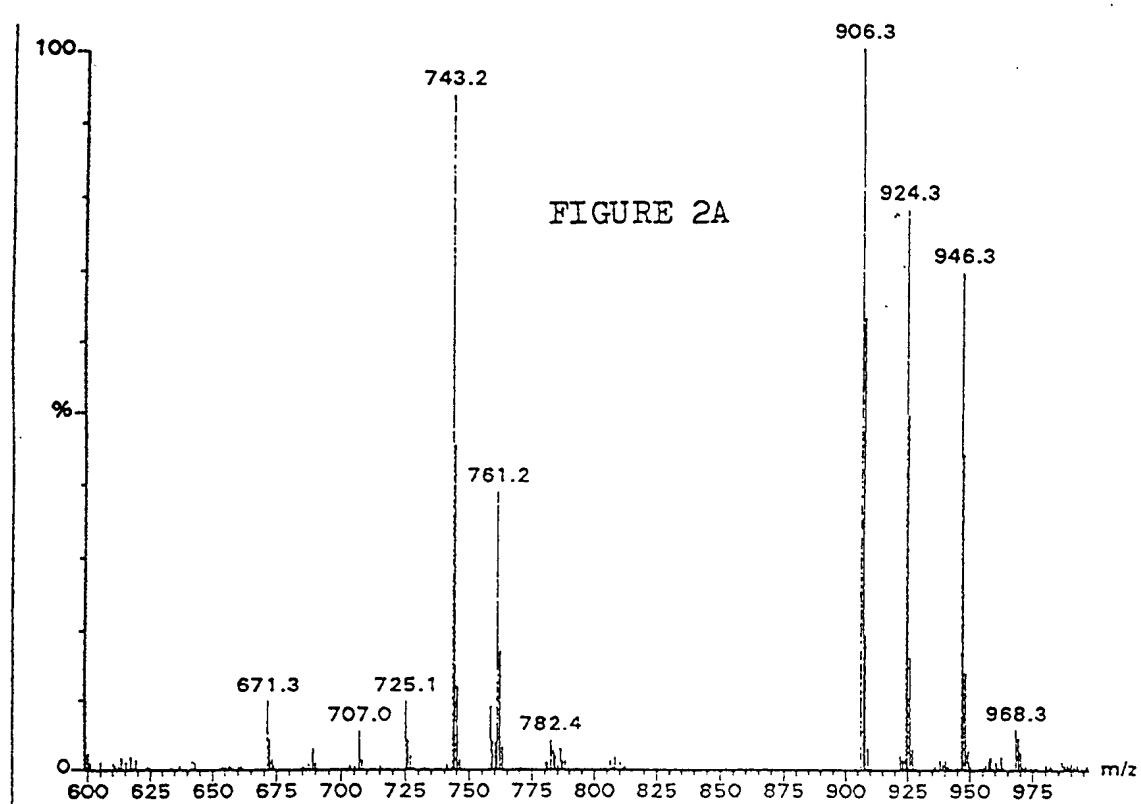
10 c) The limitation: ***wherein the molar ratio of the active ingredient : protein is within the range of 1 : 0.05 to 1 : 100, preferably 1 : 0.1 to 1 : 50 .***

15 Claim 2 as well as all claims which are dependent therefrom exclude any ratios where the protein was present in a different ratio; such as e.g. WO 98/28011 published in the priority interval. The method disclosed there was related to one single antibiotic to be used in combination with a base (e.g. 20 N-methyl-glucamine) and HSA in a molar ratio of 1 : about 2-6 : about 0.006 to 0.030.

3. All limitations are based on the statements and facts disclosed in the specification and examples.

25 4. **All claims** now on file are considered to be **novel over all citations** contained in the Search Report.

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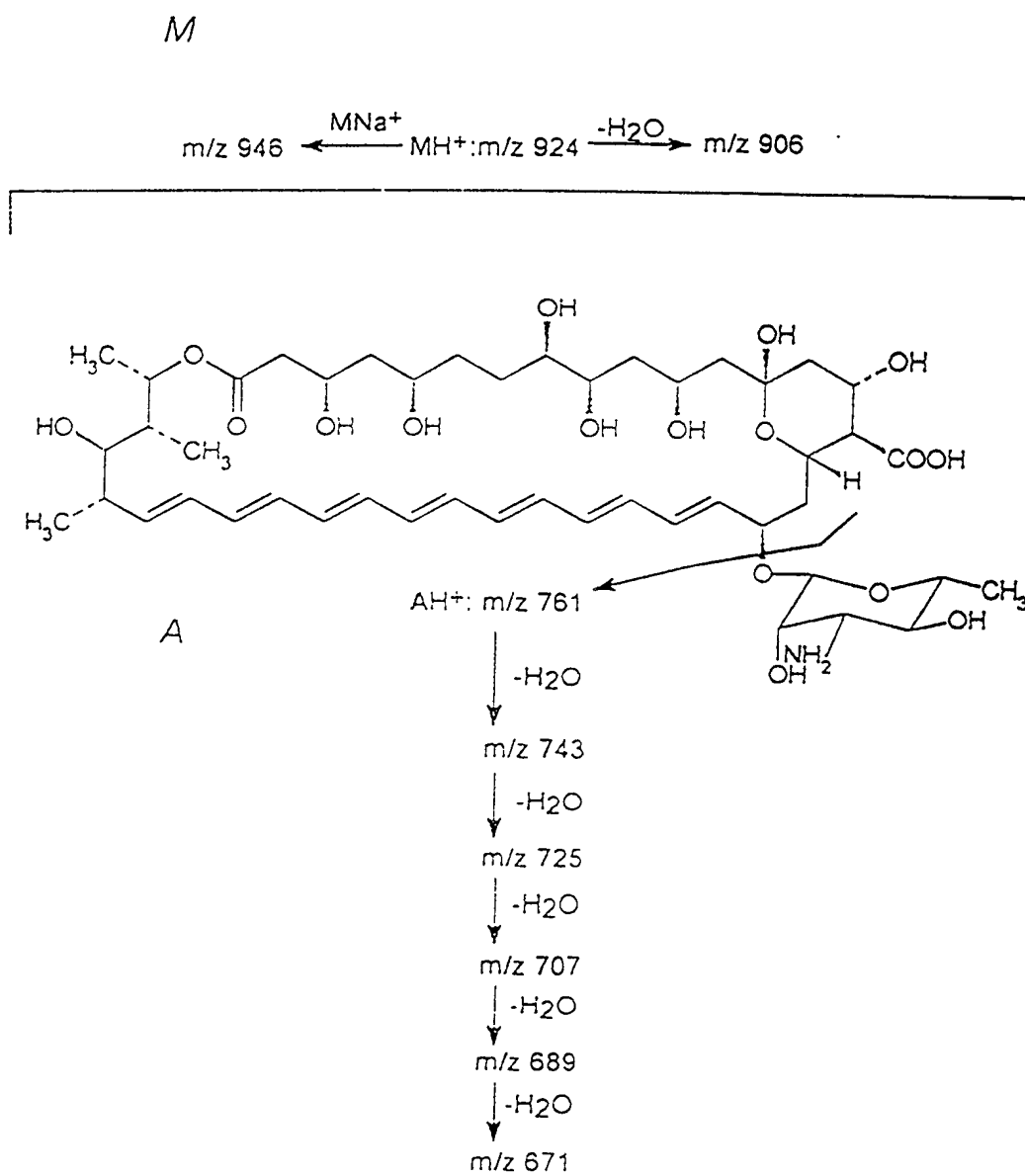
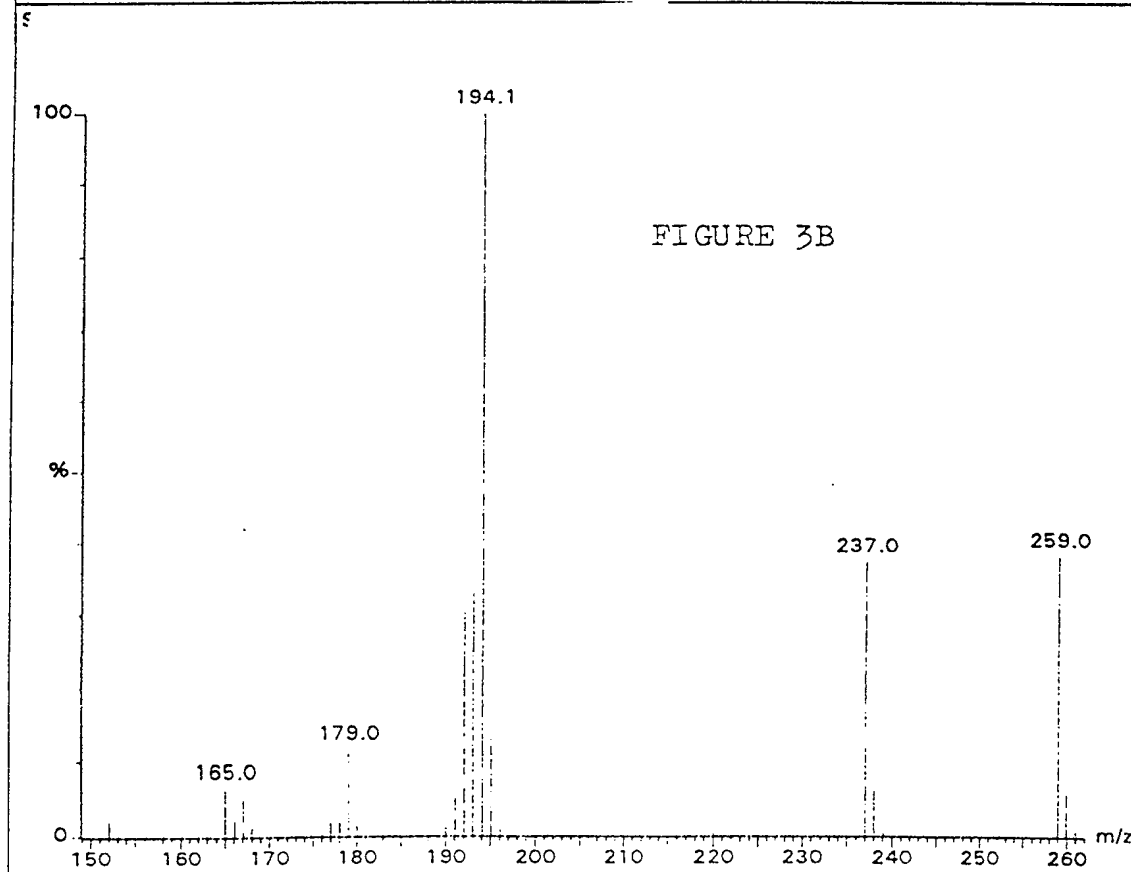
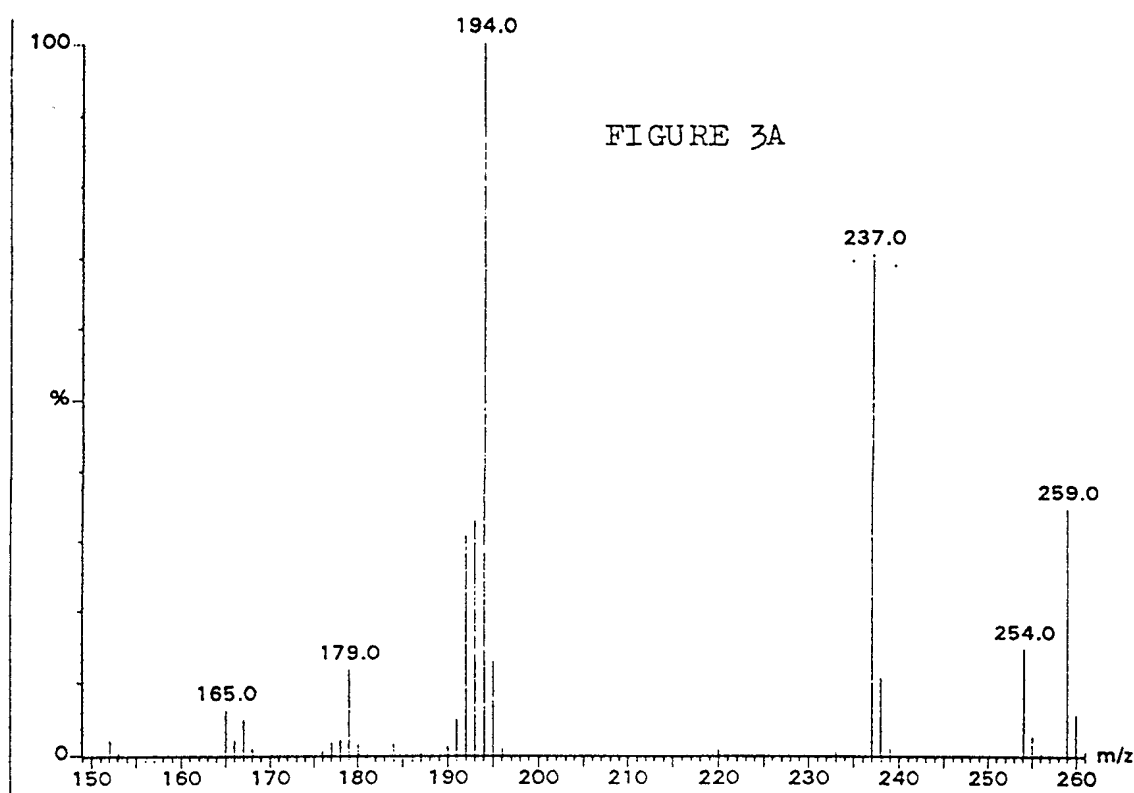


FIGURE 2C

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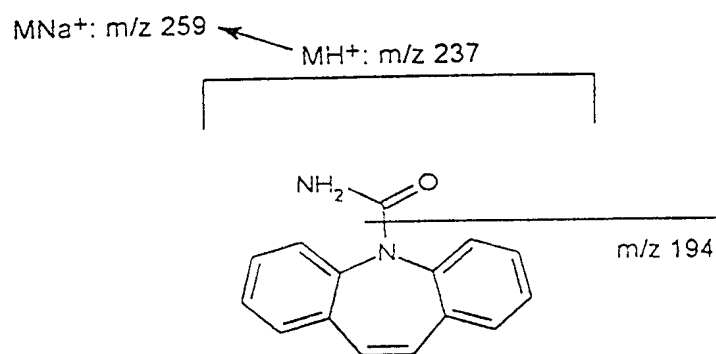
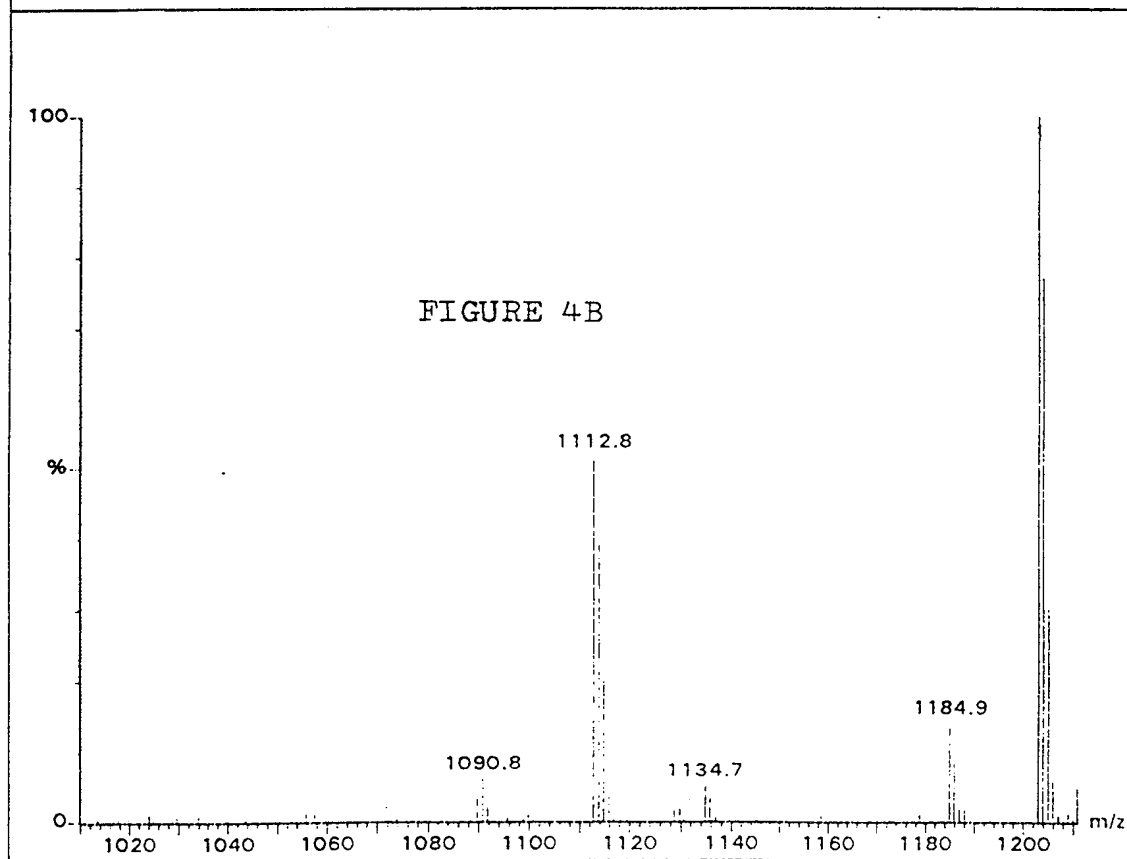
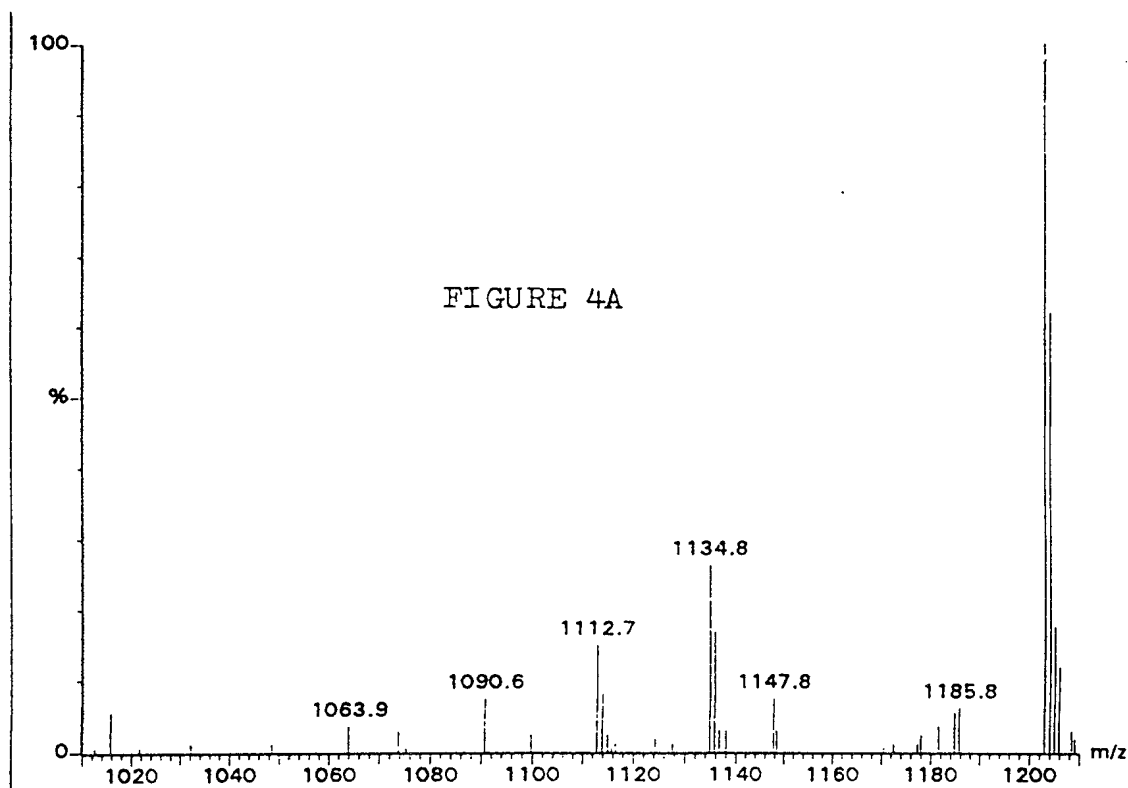


FIGURE 3C

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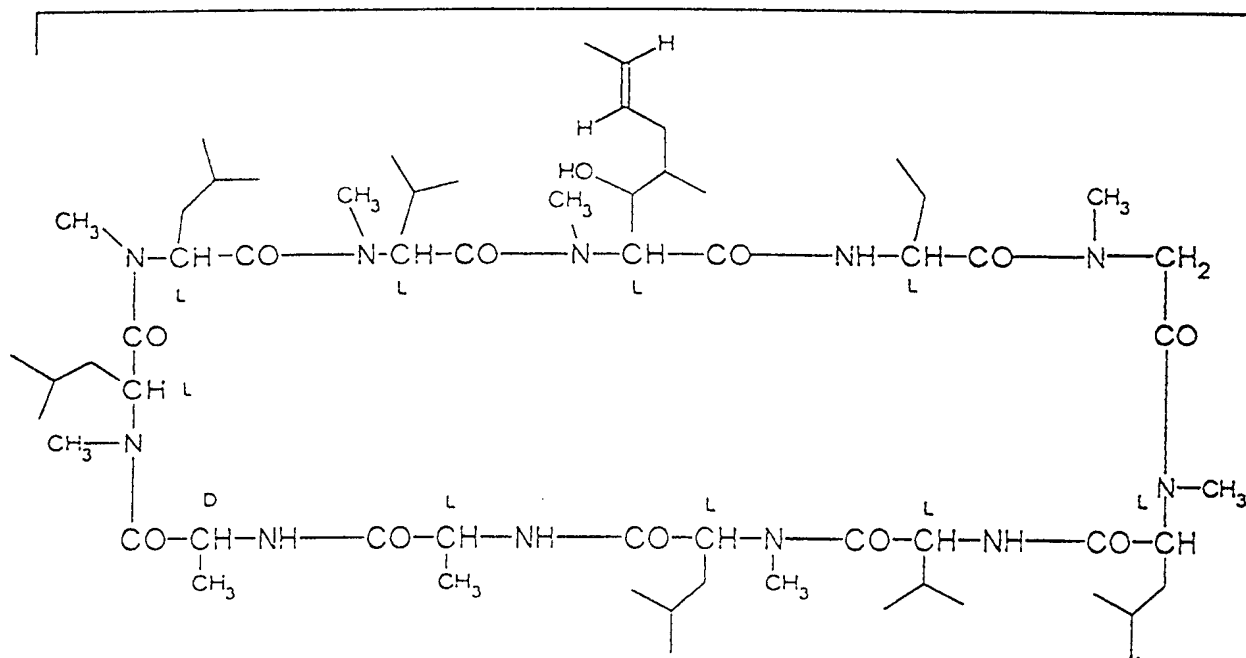
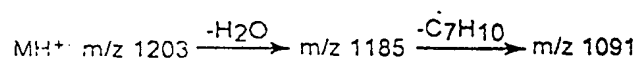
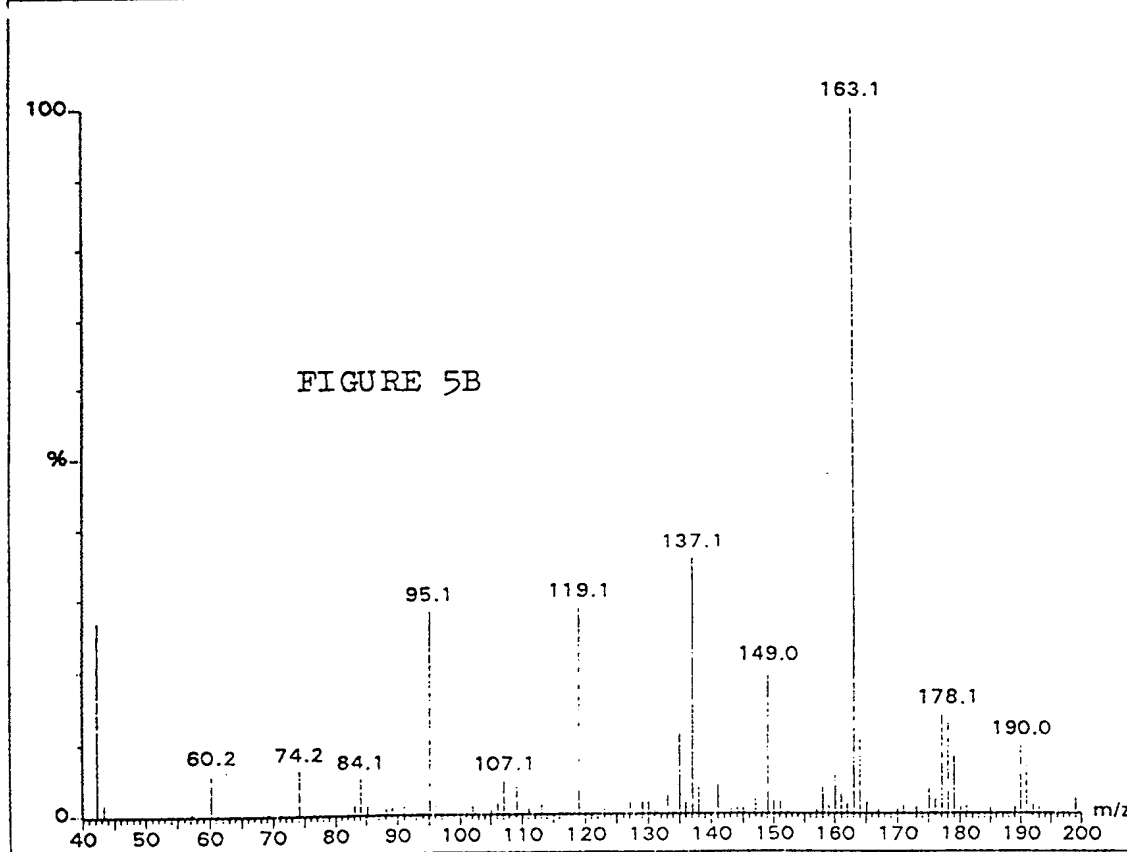
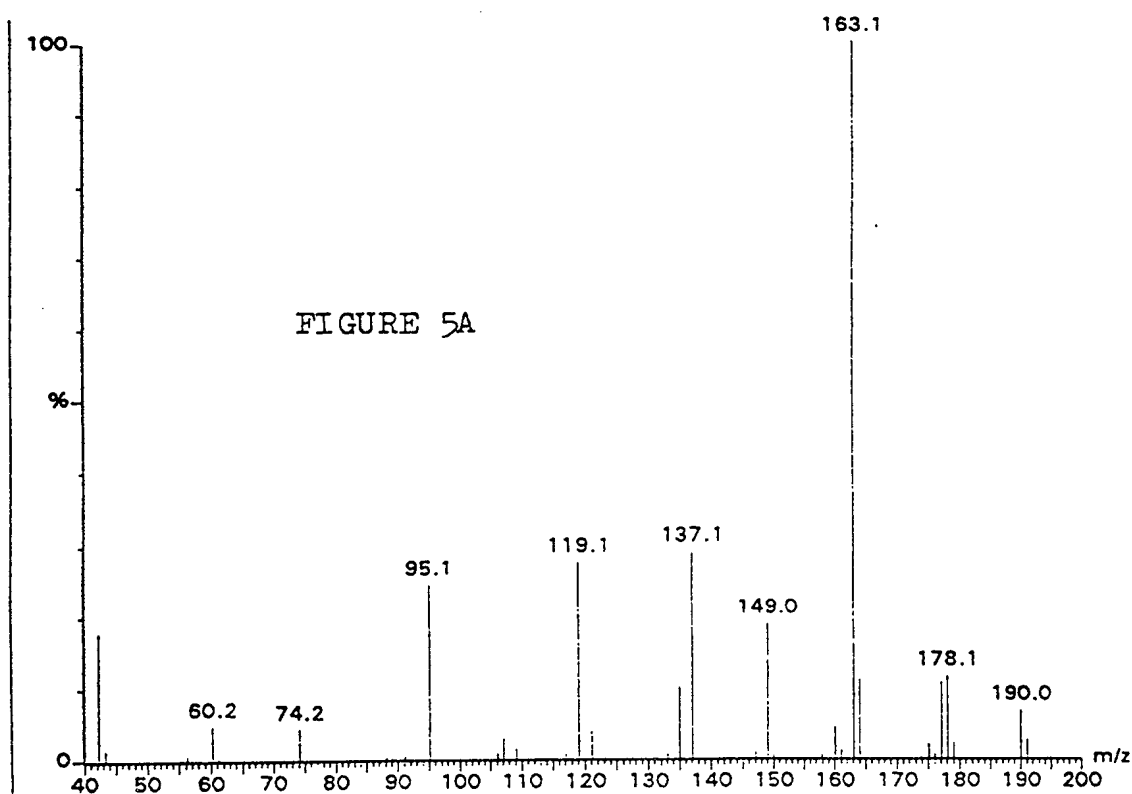
M series

FIGURE 4C

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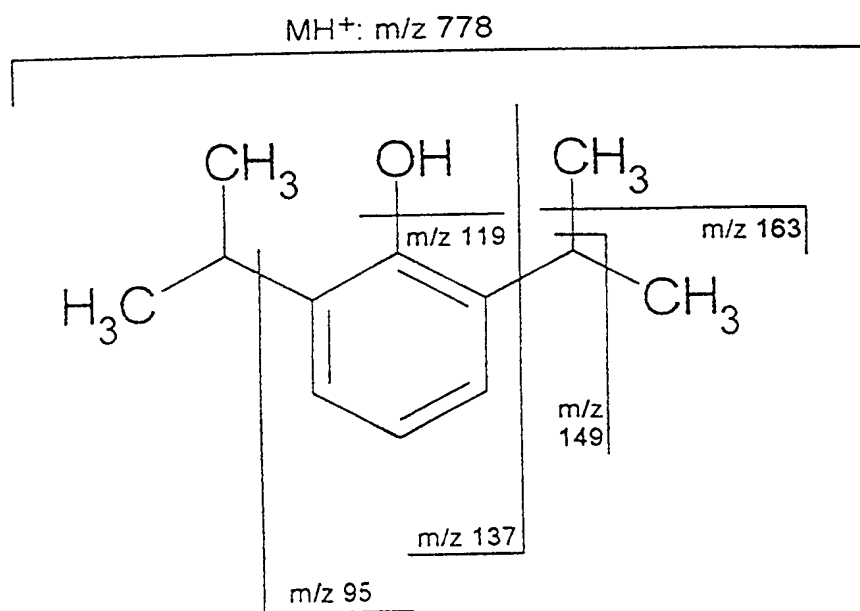
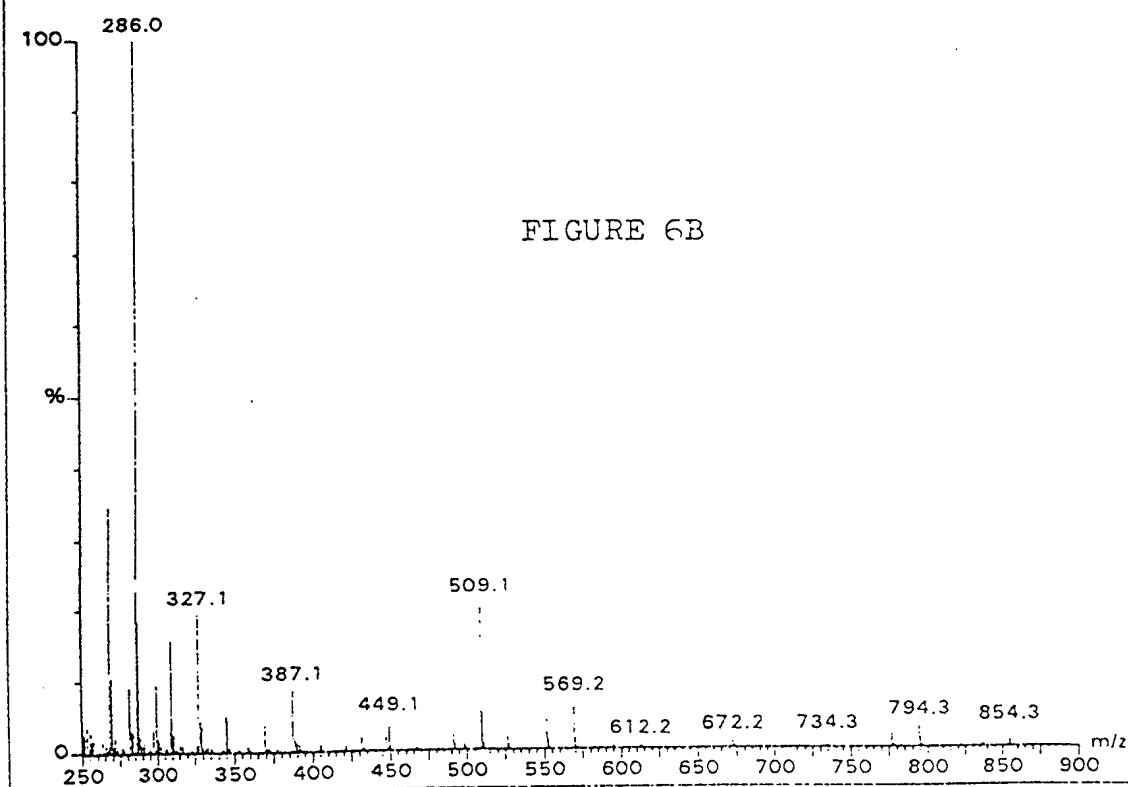
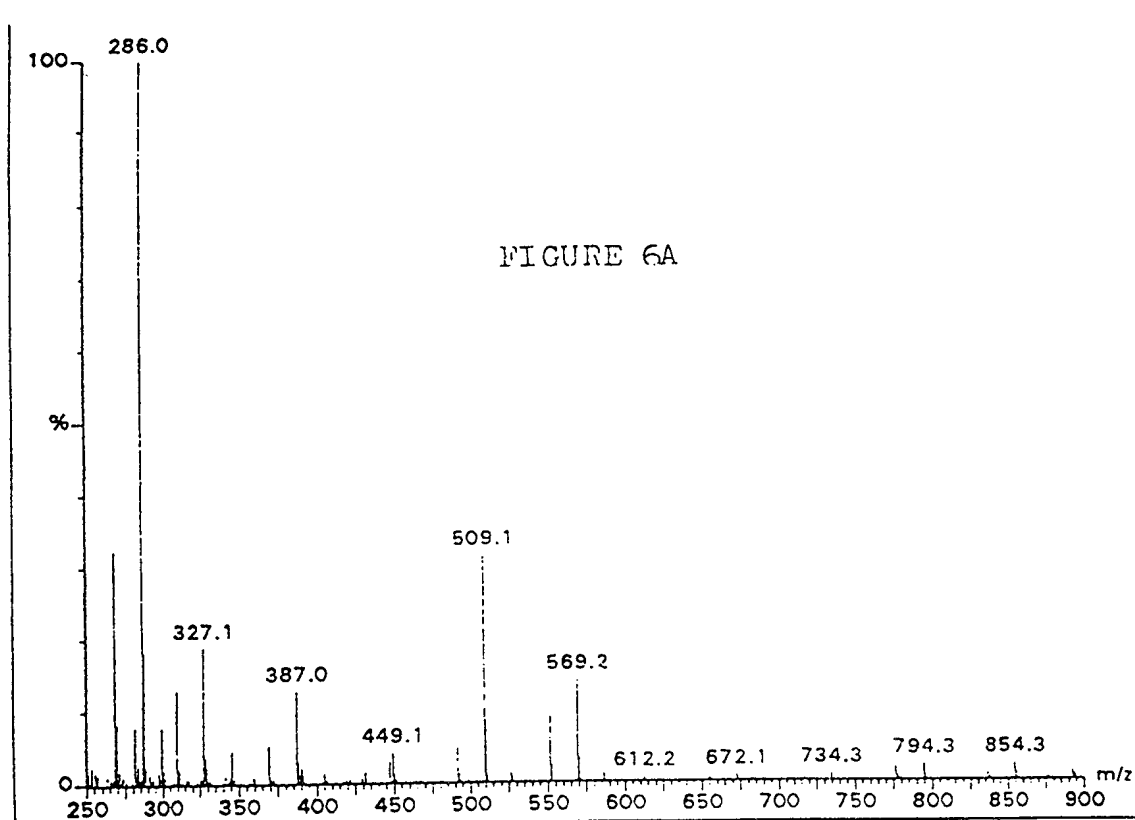


FIGURE 5C

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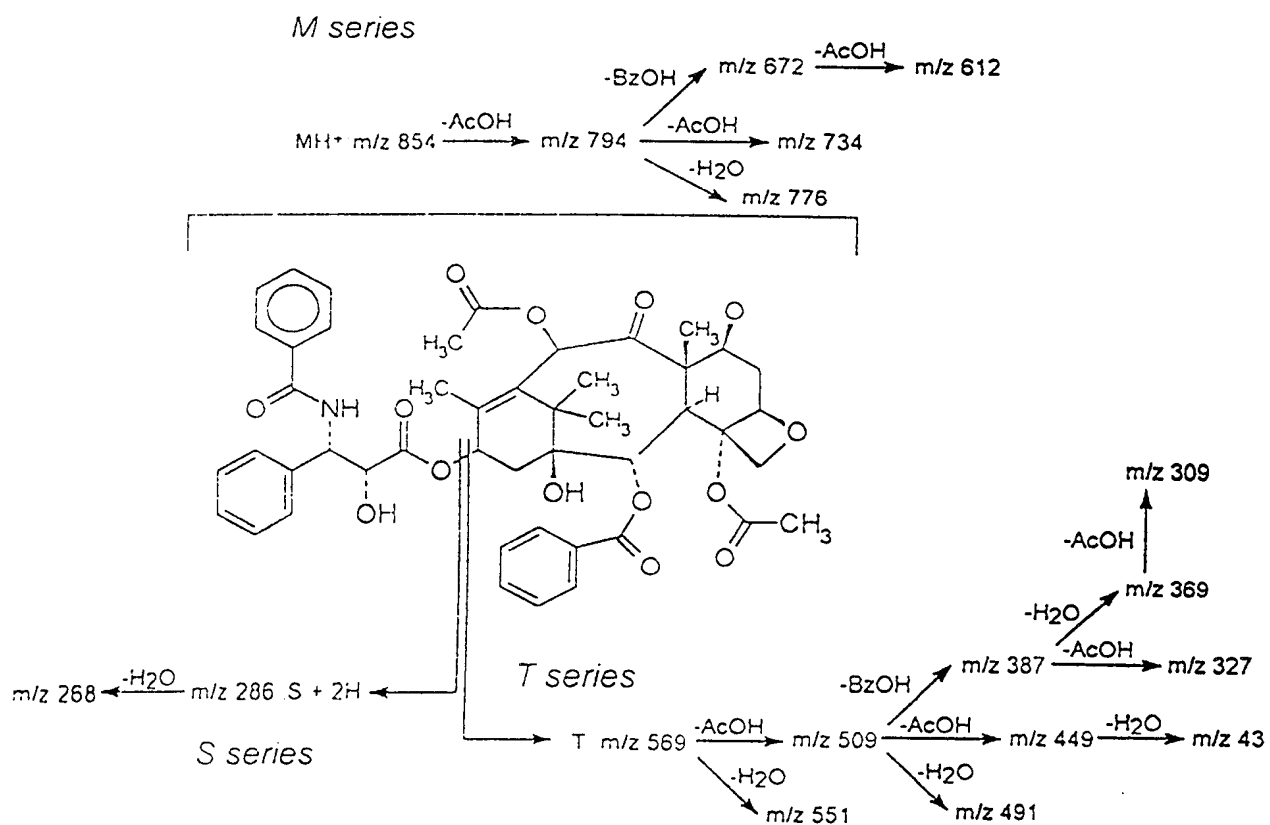


FIGURE 6C

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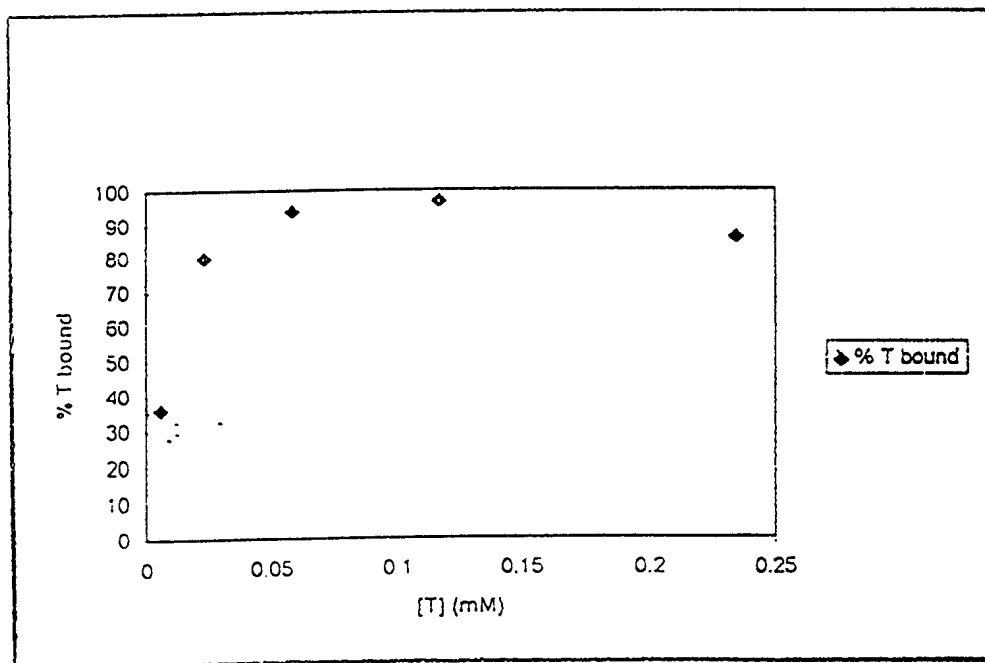
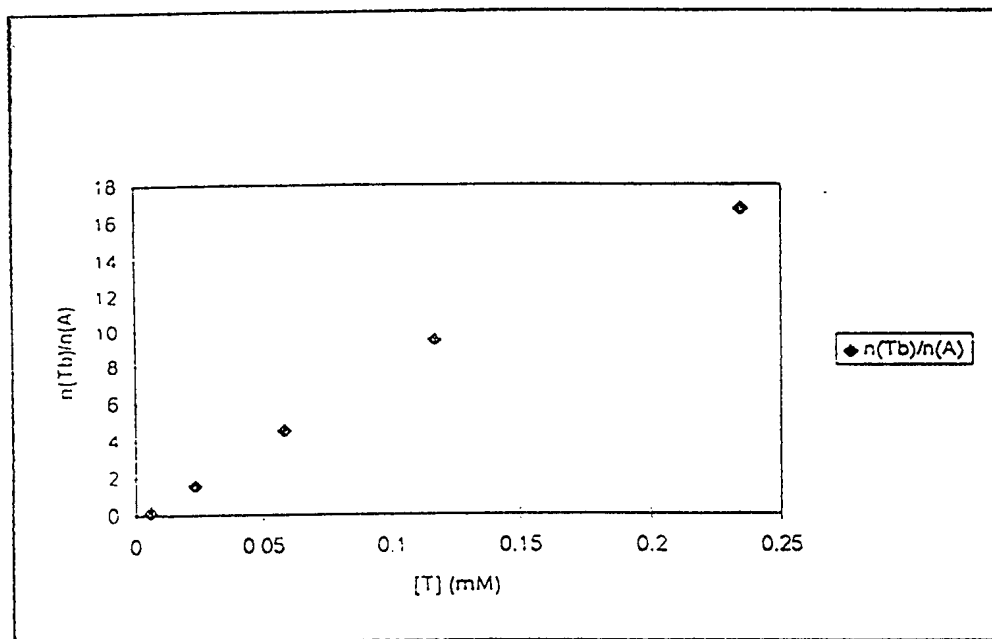


FIGURE 7

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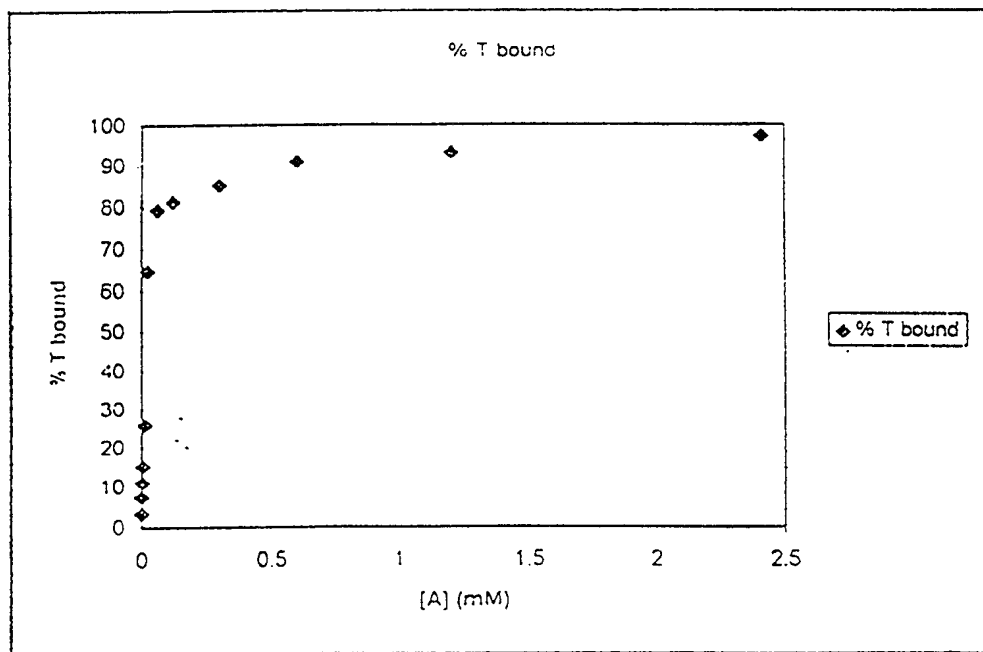
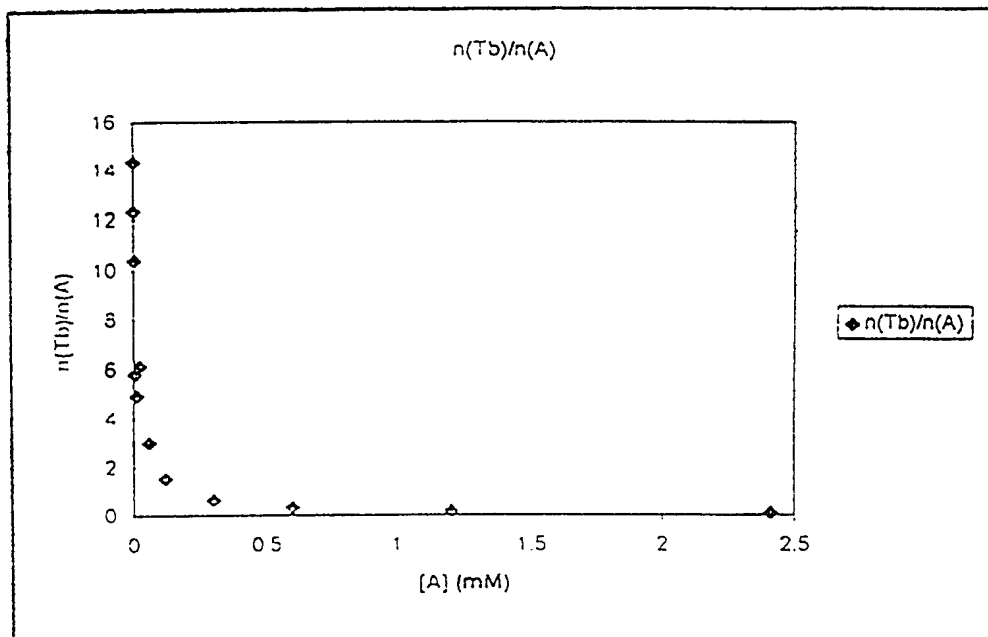


FIGURE 8

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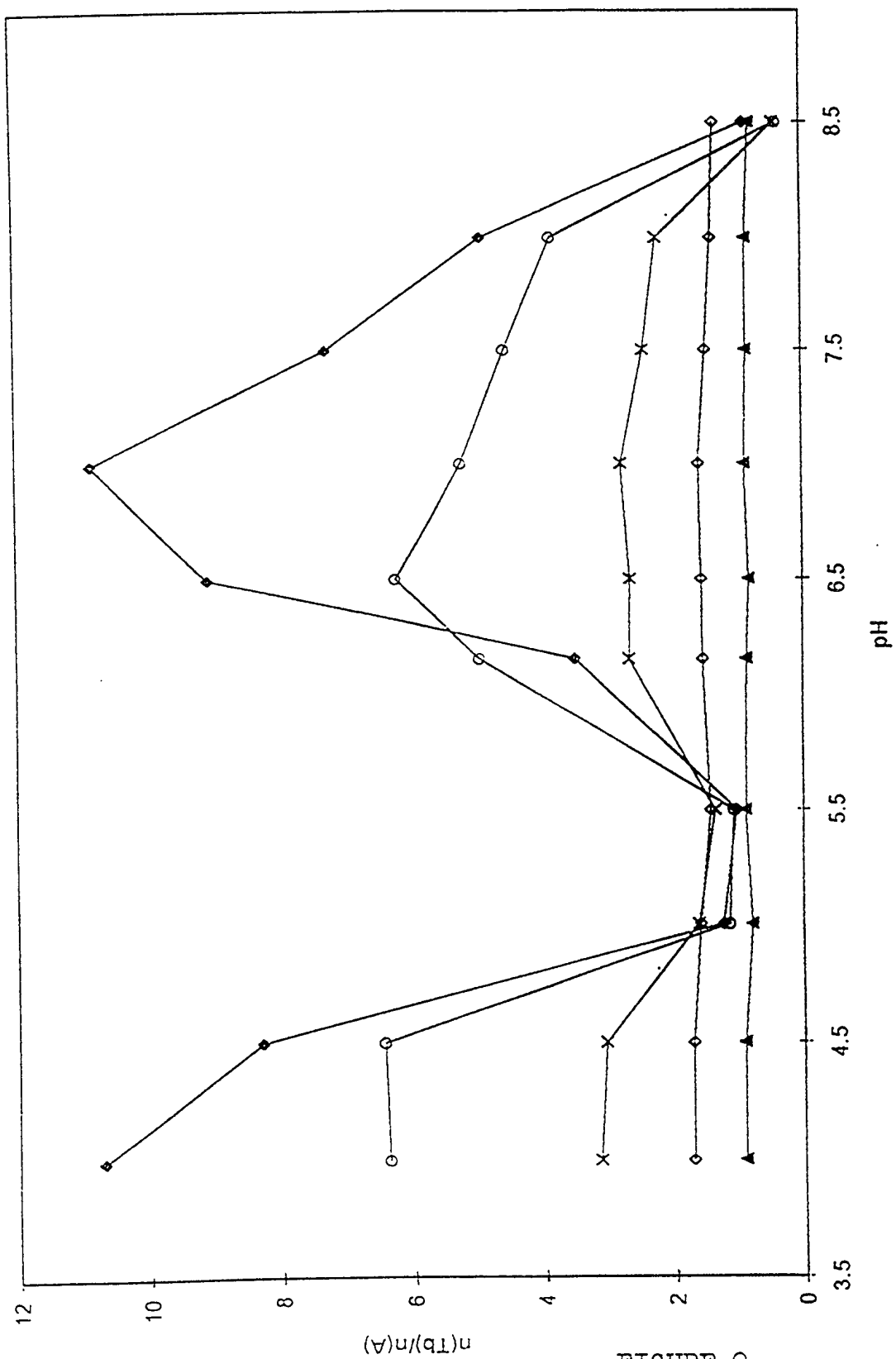


FIGURE 9

INTERNATIONAL SEARCH REPORT

national Application No
PCT/HU 98/00086

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/42 A61K9/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 326 618 A (NIPPON HYPOX LAB INC) 9 August 1989 see page 3, line 18-28 see page 5, line 12-17 see page 6, line 1 see page 7, line 3 see page 9, line 13-25 see claims 1,4	1,2,4-7, 23-28, 30,33, 35,38
X	PATENT ABSTRACTS OF JAPAN vol. 008, no. 062 (C-215), 23 March 1984 & JP 58 216126 A (ONO YAKUHI KOGYO KK), 15 December 1983 see abstract	1,2,4-7

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

5 November 1998

Date of mailing of the international search report

11/11/1998

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

La Gaetana, R

INTERNATIONAL SEARCH REPORT

national Application No
PCT/HU 98/00086

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 37 02 105 A (BAYER AG) 4 August 1988 cited in the application see claims see page 2, line 64 - page 3, line 7 ----	1,2,4
P,X	WO 98 28011 A (SCHERING CORP) 2 July 1998 see page 2, line 21-32 see page 6, line 5-10 see page 6, line 19-21 see page 6, line 37-39 see page 25, line 9-22 see example 7 see claim 1 ----	1-4
P,A	WO 98 14174 A (SOON SHIONG PATRICK ;TAO CHUNLIN (US); YANG ANDREW (US); DESAI NEI) 9 April 1998 see page 11, line 22 - page 12, line 5 see page 12, line 15-25 see page 14, line 9-23 see page 17, line 13 - page 18, line 34 see page 22, line 1 - page 28, line 37 see claims 1-7 -----	1-41

INTERNATIONAL SEARCH REPORT

International application No.

PCT/HU 98/00086

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 38-40
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 38-40
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/HU 98/00086

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